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**INDUCTION OF LINEAGE AND DIFFERENTIATION OF ADULT
HUMAN NEUROEPITHELIAL PROGENITORS IN VITRO**

By

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B.S., Nanjing University, 1989

A Dissertation submitted to the Faculty of the
Graduate School of the University of Louisville
In Partial Fulfillment of the Requirements for
The Degree of

Doctor of Philosophy

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University of Louisville, School of Medicine
Louisville, Kentucky

May 2005

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ACKNOWLEDGMENTS

I would like to sincerely thank my dissertation mentor: Dr. Fred J. Roisen for his insightful guidance and support throughout my Ph.D. education. I also acknowledge my other committee members: Drs. Kathleen Klueber, Mengsheng Qiu, Peter Rowell, Scott Whitemore for their thoughtful suggestions. Furthermore, I would like to thank Dr. Chengliang Lu, Dr. Jun Cai, Dr. Nigel Cooper, Dr. Michal Hetman, Zhanfang Guo, Ming Xiao, Manal Othman, Charles Marshall, George Harding, and Cathie Caple for their continued support over the past few years. Finally, I am indebted to my parents: Zhaoqun Zhang and Huizheng Tang, and my wife Zhanfang Guo, and my son Zixiang Zhang for their continued encouragement and support.

ABSTRACT

INDUCTION OF LINEAGE AND DIFFERENTIATION OF ADULT HUMAN NEUROEPITHELIAL PROGENITORS IN VITRO

Xiaodong Zhang

April 26, 2005

Neurosphere forming cells (NSFCs) have been derived from cultures of adult olfactory neuroepithelium obtained from patients and cadavers in serum rich medium. These neural progenitors remain undifferentiated when maintained in serum rich medium but have the potential to differentiate along glial or neuronal lineages. A totally defined medium (DM) was employed to examine their proliferation, lineage restriction and differentiation. None of the neurotrophic factors evaluated increased NSFCs viability or lineage restriction under the defined experimental conditions. Few of NSFCs ever express mature neuronal or glial markers in DM.

To evaluate the potential of NSFCs to form oligodendrocytes, transcription factors, *Olig2*, *Nkx2.2*, and *Sox10*, were introduced into NSFCs to determine if their expression is sufficient for oligodendrocyte differentiation. Simultaneous transfection of *Olig2-Nkx2.2*, or *Nkx2.2-Sox10* cDNA produced characteristic oligodendrocyte morphology and antigenicity including myelin basic protein. Furthermore, a population of *Olig2*-expressing NSFCs also expressed *Sox10*. Coculture of NSFCs

transfected with *Olig2-Nkx2.2*, or *Nkx2.2-Sox10* with purified sensory neurons demonstrated ensheathment formation between NSFC processes and axons.

Retinoic acid, forskolin, and sonic hedgehog (RFS) have been reported to play important roles in neurogenesis in embryonic CNS *in vivo*. The application of RFS to NSFCs induced a small population to express mature neuronal antigens and to undergo neurite formation. To further increase the neuronal, especially motoneuronal, antigen expression, transcription factors *Olig2*, *Ngn2* and *HB9* were introduced into NSFCs to determine if their expression was sufficient for motoneuron differentiation. Simultaneous transfection of *Ngn2-HB9* or *Olig2-HB9* cDNA, supplemented with RFS produced increased expression of motoneuron antigens. Furthermore, high levels of *Olig2* expressing NSFCs also expressed *Ngn2*. Coculture of NSFCs transfected with *Ngn2-HB9*, or *Olig2-HB9* with purified chicken skeleton muscle cells demonstrated the formation of neuromuscular junctions.

Our long-term goal is to develop cell populations for future studies that would be employed to determine the therapeutic utility of these olfactory-derived NSFCs for autologous transplantation into donors with CNS trauma and neurodegenerative diseases as well as for use in diagnostic evaluation.

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CHAPTER I

INTRODUCTION

Adult human olfactory neuroepithelial progenitors

Olfactory epithelium is located in the olfactory mucosa in the upper part of nasal cavity. It is pseudostratified columnar epithelium, which mainly consists of olfactory receptor neurons, sustentacular cells, and basal cells. Olfactory receptor neurons are bipolar neurons, their nuclei are located in the middle region of the olfactory epithelium. Olfactory receptor neurons carry the signal following chemoreception through their receptors to the olfactory bulb where they synapse with mitral cells in the glomeruli of the olfactory bulb (Dodd and Castellucci, 1991). The life span of the olfactory receptor neurons is about 30-40 days after which they are replaced (Caggiano et al., 1994; Graziadei and Graziadei, 1979a, 1979b; Moulton, 1974). The sustentacular cells are columnar cells, their nuclei are located in the most superficial region of the olfactory epithelium. The sustentacular cells give metabolic and physical support to the olfactory receptor neurons (Ross et al., 1995). The basal cells are located in the base of the olfactory epithelium. There are two types of basal cells: globose basal cells that give rise to neurons in the olfactory epithelium, and horizontal basal cells that give rise to the globose basal cells in the olfactory epithelium throughout life (Moulton, 1974; Graziadei and Graziadei, 1979a; 1979b; Ross et al., 1995; Satoh and Takeuchi, 1995).

Currently, substantial efforts are aimed at inducing neural stem cells to differentiate into motoneurons and oligodendrocytes. Neural stem cells have been reported to differentiate into neurons, astrocytes and oligodendrocytes after exposure to PDGF, CNTF, and T3 (Dietrich, et al., 2002; Rao and Mayer-Proschel, 1997). Neural stem cells from the striata of adult mice proliferated and formed neurospheres in serum-free culture medium with the addition of epidermal growth factor (EGF) (Reynolds and Weiss, 1992). This is the first demonstration of neurosphere formation *in vitro*. The neurosphere is composed of proliferating cells which remain undifferentiated, and can generate both neurons and glia after the withdrawal of mitogens EGF or bFGF (Reynolds et al., 1992; Vescovi et al., 1993). Embryonic rat striatal stem cells, proliferated in serum-free culture medium but did not form neurospheres in the presence of nerve growth factor (NGF) and basic fibroblast growth factor (bFGF) (Cattaneo and McKay, 1990). An understanding of factors contributing to the modulation of these cells *in vitro* will aid in the development of procedures for obtaining novel populations of progenitor cells for transplantation into the diseased or damaged central nervous system or for use in drug discovery and pharmacological evaluation.

Olfactory neuroepithelium contains neural stem cells, which can regenerate and give rise to olfactory receptor neurons and supporting cells throughout the life (Calof and Chikaraishi, 1989; Sosnowski et al., 1995; Zhang et al., 2000; Roisen et al., 2001; Zhang et al., 2001). Stem cells from the olfactory neuroepithelium provide a unique source of adult neural progenitor cells, which can be obtained from patient's own olfactory neuroepithelium, and may someday be used for autologous transplantation.

Autologous transplantation would eliminate the need for immunosuppression and thus avoid graft-host rejection. Human olfactory neuroepithelium is located within the nasal cavity and can be obtained by endoscopic biopsy during nasal sinus surgery without causing damage to the donor (Winstead et al., 2005). Therefore, it is possible to obtain these cells for autologous transplantation without highly invasive surgery. Previously, our lab has shown that cultures of olfactory epithelium can be grown to yield a population of highly mitotic neural progenitors (Roisen et al., 2001). These cells and their subcultures form neurospheres and can be maintained for extended periods in media supplemented with 10% fetal bovine serum. The development of a totally defined medium that would support their long-term growth is an essential technical step in the further characterization of these neurosphere forming cells (NSFCs).

Olfactory neuroepithelium consists of olfactory receptor neurons, supporting (sustentacular) and basal cells that have been shown to be responsible for replacement of damaged cells throughout life (Calof and Chikaraishi, 1989; Sosnowski et al., 1995; Liu et al., 1998; Zhang et al., 2000; Zhang et al., 2001). The unique regenerative capacity of this tissue suggested that stem cells could be isolated from olfactory neuroepithelium. *In vitro* they produced a population of neurosphere forming cells (NSFCs). To date the lab has been successful in isolating NSFCs from adult postmortem olfactory neuroepithelium (Roisen et al., 2001) as well as from patients undergoing endoscopic nasal sinus surgery (Winstead et al., 2005). In 10% of the primary cultures of olfactory neuroepithelium established in minimum essential medium (MEM) with Hanks and 10% heat-inactivated fetal bovine serum, a

population of NSFCs was obtained within 8 weeks (Roisen et al., 2001). Under these culture conditions, the NSFCs expressed lineage specific markers including β -tubulin isotype III, NCAM and microtubule-associated-protein-2 (MAP2) as well as the protein nestin. A few cells were immunopositive for A2B5 and GFAP, but negative for p75^{NTFr} (Roisen et al., 2001). The survival and proliferation of the NSFCs required MEM supplemented with 10% FBS. However, the composition of serum is complex and variable. In addition, the quality of the serum varies from batch to batch. The presence of factors contained within serum could mask the effects of growth factors on the cells as well as affect their growth and differentiation, thus making characterization of the NSFCs difficult. Other neuronal precursor cells including embryonic quail neural crest cells (Langtimm-Sedlak et al., 1996), rat neuroepithelial cells (Maric et al., 2000) and cortical neural precursor cells (O'Connor et al., 2000) have been successfully cultured in serum-free medium. The first objective of this thesis was to determine if adult olfactory neuroepithelial progenitors could be maintained, propagated and differentiated in a serum-free defined medium. Ultimately, a defined medium would be used to characterize the NSFC requirements for growth factors and trophic support to develop populations of adult human olfactory-derived neural stem cells as a source for transplantation into the injured central nervous system. These cells could be used for neuroreplacement therapy without the ethical dilemma associated with use of fetal tissue or the need for immunosuppression (Roisen et al., 2001).

Oligodendrocytic development

Oligodendrocytes are the myelin-forming cells in the central nervous system (CNS), and may also regulate the microenvironment around neurons (Ludwin, 1997). Populations of oligodendroglia could perhaps be used for therapeutic cell replacements in myelin diseases (Scherer, 1997; Duncan et al., 1997).

During development, oligodendrocytes arise from the glial precursors, which are derived from the ventral neural tube (Warf et al., 1991; Noll and Miller, 1993; Ono et al., 1995; Orentas and Miller, 1999; Richardson et al., 2000). The ventral midline signal protein sonic hedgehog (Shh) has been shown to induce oligodendrocyte formation in dissociated and explant rodent cultures (Trousse et al., 1995; Roelink et al., 1995; Poncet et al., 1996; Pringle et al., 1996.). The chimeric protein containing soluble interleukin-6 (IL-6) receptor and IL-6 fusion protein induced new born rat oligodendrocyte progenitor cells to differentiate into the mature oligodendrocytes (Valerio et al., 2002); CNTF and LIF promoted the differentiation of early oligodendrocyte progenitors from embryonic rat towards mature oligodendrocytes (Mayer et al., 1994); and erythropoietin (Epo) promoted differentiation and/or maturation of oligodendrocytes from embryonic rat cerebral hemispheres (Sugawa et al., 2002). In contrast, bone morphogenetic proteins (BMPs) inhibited the generation and differentiation of oligodendroglia from cultures of embryonic rat (Zhu et al., 1999, Mabie et al., 1999), postnatal rat (Mabie et al., 1997), and embryonic mouse (Gross et al., 1996). Thus, many factors affect oligodendrocyte maturation.

In the developing chicken and mouse CNS, *Olig1* and *Olig2* genes encoding basic helix-loop-helix (bHLH) protein, were expressed in oligodendrocyte-generative

zones of the neuroepithelium and in oligodendrocyte progenitors (Zhou et al., 2000; Lu et al., 2000; Qi et al., 2001). Olig2 expression persists in mature oligodendrocytes from the embryonic rat and chick (Lu et al., 2000; Zhou et al., 2001). Thus, Olig2 plays an important role in oligodendrocyte lineage determination *in vivo*. *Nkx2.2* gene encoding homeodomain (HD) transcription factor is also specifically expressed in oligodendrocyte progenitors from embryonic chick (Xu et al., 2000; Fu and Qiu, 2001; Qi et al., 2001; Zhou et al., 2001) and embryonic rodent (Qi et al., 2001) spinal cord and brain. Mutation of the *Nkx2.2* gene caused a decrease in the number of mature oligodendrocytes in the spinal cord and brain (Xu et al., 2000; Qi et al., 2001). Thus, *Nkx2.2* regulates the progression of oligodendrocyte progenitors from the embryonic chicken and rodent to later stages of differentiation *in vivo* (Qi et al., 2001; Zhou et al., 2001). In culture systems, embryonic stem cells of neural lineage were shown to have morphological and antigenic properties of oligodendrocyte lineage when the transcription factor Olig2 was inserted (Xian et al., 2003). *In vivo*, coelectroporation *Olig2* and *Nkx2.2* promoted embryonic chicken and mouse oligodendrocyte differentiation and maturation (Zhou et al., 2001; Qi et al., 2001). The second objective of this thesis was to investigate the roles of *Olig2* and *Nkx2.2* genes in human oligodendrocyte lineage specification and differentiation *in vitro* using adult human olfactory neuroepithelial progenitors.

Neuronal development

Olig2 has been shown to be essential in the generation of oligodendrocytes and motoneurons *in vivo* (Novitsch et al., 2001). Olig2 is expressed by motoneuron progenitors, has a key role in specifying pan-neuronal properties of developing

motoneurons, precedes the expression of MNR2 and is rapidly extinguished in post-mitotic motoneurons. Olig2 also directs expression of motoneuron transcription factors including Islet1/2 (Isl1/2), MNR2/HB9 in neural progenitor cells (Novitch et al., 2001). Ngn2 is required for the generation of mouse motoneurons (Scardigli et al., 2001), the development of the mouse cranial sensory ganglia (Fode et al., 1998), and functions as a vertebrate neuronal lineage factor (Ma et al., 1996; Farah et al., 2000). Ngn2 and NeuroM coupled with Isl1 and Lhx3 specified motoneurons in the embryonic chicken spinal cord and mouse P19 embryonic carcinoma cells (Lee and Pfaff, 2003). Olig2 and Ngn2 were specifically coexpressed in motoneuron progenitor cells, and resulted in cell cycle exit and pan-neuronal properties of developing chicken motoneuron differentiation (Mizuguchi et al., 2001; Novitch et al., 2001). Lineage-restriction transcription factors HB9/MNR2 are specifically expressed in all somatic motoneurons. MNR2 is expressed in presumptive motoneuron progenitor cells, and HB9 only in postmitotic motoneurons in the chick; HB9 is expressed in presumptive motoneuron progenitor cells and maintained expression in motoneurons in the mouse (Tanabe et al., 1998; Arber et al., 1999; Thaler et al., 1999; Odden et al., 2002; William et al., 2003). Transcription factor HB9 is evolutionarily conserved, expressed selectively by embryonic motoneurons, and is essential for motoneuron development and differentiation (Lee and Pfaff, 2003). Coexpression of Ngn2 and MNR2 increased the number of Isl1/2 [Isl1/2 and HB9: defined markers for positive motoneurons and their progenitors (Mizuguchi et al., 2001)] in the chicken spinal cord compared with the MNR2 alone (Novitch et al., 2001). Mature motoneurons are characterized by cytoskeletal components, their ability to release neurotransmitters,

their surface receptors and their ability to form synapses (Reh, 2002; Song et al., 2002). Retinoic acid (RA), forskolin (FN), and sonic hedgehog (Shh) regulate neuronal specification and differentiation (Roisen et al., 1972a; 1972b ; Roelink et al., 1994, 1995; Ericson et al., 1997; Sockanathan and Jessell, 1998; Maden, 2002; Novitch et al., 2003; Diez del Corral et al., 2003; Bibel et al., 2004; Perrier et al., 2004). RA and Shh have been shown to determine the expression of HD and bHLH transcription factors which act on neuronal fate, especially motoneurons in the ventral spinal cord and mouse embryonic stem cells (Novitch et al., 2003; Diez del Corral et al., 2003; Wichterle et al., 2002; Sockanathan et al., 2003). The third objective of this thesis was to investigate the roles of *Olig2*, *Ngn2*, and *HB9* genes and RA, FN, and Shh in human neuronal lineage specification and differentiation *in vitro* using adult human olfactory neuroepithelial progenitors. In this thesis the lineage potential of NSFCs has been examined and extended with transcription factors and agents known to direct neural progenitor differentiation.

CHAPTER II
ADULT HUMAN OLFACTORY NEURAL PROGENITORS CULTURED IN
DEFINED MEDIUM

Xiaodong Zhang, Kathleen M. Klueber, Zhanfang Guo, Chengliang Lu
and Fred J. Roisen

Published in Experimental Neurology, 2004, 186(2):112-123.

Abbreviations:

ACS, aqueous counting solution; BCA, BicinHoninic acid; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; BSA, bovine serum album; CB, cytoskeletal buffer; CNS, central nervous system; CNTF, ciliary neurotrophic factor; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; DF, DMEM/F12 and 10 mg% gentamycin; DF2.5, DF with 2.5% FBS; DFB27, DF with 2% B27; DFInsu, DF and 10 µg/ml human insulin; DFN2, DF with 1% N2; DIV, days in vitro; DM, defined medium; EGF, epidermal growth factor; FBS, heat-inactivated fetal bovine serum; GFAP, glial fibrillary acidic protein; IGF, insulin-like growth factor; MAP2, microtubule-associated-protein-2; MEM, minimum essential medium; MEM2.5, MEM with 2.5% FBS, 10 mg% gentamycin; MEM10, MEM with 10% FBS, 10 mg% gentamycin; MGS, multinucleated giant cells; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NCAM, neural cell adhesion molecule; NGF, nerve growth factor; NSFCs, neurosphere-forming cells; NT-3, neurotrophic factor 3; NTFs, neurotrophic factors; ODC, ornithine decarboxylase; OE, olfactory epithelium; PDGF, platelet derived growth factor; TBS, Tris-Buffered Saline.

INTRODUCTION

Olfactory neuroepithelium (OE) consists of olfactory receptor neurons, supporting cells, and basal stem cells that have been shown to be responsible for replacement of damaged cells throughout life (Calof and Chikaraishi, 1989; Sosnowski et al., 1995; Zhang et al., 2000; 2001). The unique regenerative capacity

of this tissue suggests that stem cells isolated from OE might be cultured to produce a population of neurosphere forming cells (NSFCs). Our laboratory has been successful in isolating NSFCs from adult postmortem OE (Roisen et al., 2001; 2002a) as well as from patients undergoing endoscopic nasal sinus surgery (Winstead et al., 2005). In 10% of the primary cultures of the OE established in MEM with Hanks and 10% heat-inactivated fetal bovine serum (FBS), a population of NSFCs was obtained within 8 weeks (Roisen et al., 2001). Under these culture conditions, the NSFCs expressed lineage-specific markers including β -tubulin isotype III, neural cell adhesion molecule (NCAM) and microtubule-associated-protein-2 (MAP2) as well as the protein nestin. A few cells were immunopositive for a ganglioside found in glial membranes, A2B5, and glial fibrillary acidic protein (GFAP), but negative for p75^{NGFr} (Roisen et al., 2001). The survival and proliferation of the NSFCs required MEM supplemented with 10% FBS. However, the composition of serum is complex and variable. The presence of serum could mask the effects of growth factors on the cells as well as affect their growth and differentiation, thus making characterization of the NSFCs difficult.

Other neuronal precursor cells including embryonic quail neural crest cells (Langtimm-Sedlak et al., 1996), rat neuroepithelial cells (Maric et al., 2000) and cortical neural precursor cells (O'Connor et al., 2000) have been successfully cultured in serum-free medium. The objective of the current study was to determine if adult olfactory neuroepithelial progenitors could be maintained, propagated and differentiated in a serum-free defined medium (DM). Ultimately, a DM would be used to characterize the NSFCs requirements for growth factors and trophic support.

These populations of adult human olfactory-derived neural stem cells could then be used for transplantation into the injured central nervous system (CNS), thus eliminating the ethical dilemma associated with use of fetal tissue or the need for immunosuppression (Deacon et al., 1998; Shihabuddin et al., 1999; Whittemore and Snyder, 1996).

In this study, the adult olfactory NSFCs obtained from both postmortem and living patients were maintained in DM for periods up to 1 year. The phenotypic expression of the NSFCs cultured in various supplements and different defined media was evaluated. With the reduction of serum and the addition of N2 or B27 supplement or insulin, there was a change in expression of the lineage restriction markers, which included an increase in the presence of A2B5 and other glial markers, as well as the expression in some cells of more mature neuronal markers. These changes were accompanied by a corresponding decrease in nestin positive cells.

MATERIALS AND METHODS

Cell culture

The NSFCs used in this study were obtained from frozen stock from two patients representing different sources cadaver-derived adult OE (Roisen et al., 2001) and sinus surgery patient-derived OE (Winstead et al., 2005). The NSFCs from a 96-year-old male cadaver and a 22-year-old female patient lines were cultured in MEM10 (MEM with 10% FBS, 10 mg% gentamycin) in the flasks (25 cm² surface area, Corning Incorporated, Corning, NY) in a humidified atmosphere of 5% CO₂-95% air at 37°C. Medium changes were performed every 2 days. The NSFCs were adapted to the absence of serum over 2 weeks through a series of dilutions (10%, 5%,

2.5%, 1.25%, 0.625% to 0% serum) that decreased the serum concentration by half every 2 days until the cells were cultured in defined media. Since the cells would not survive in MEM alone (data not shown), they were placed in defined media, which consisted of DF (DMEM/F12 with 10 mg% gentamycin), DFN2 (DF with 1% N2), or DFB27 (DF with 2% B27). In addition, cells grown in DFN2 were taken through a series of dilutions from 1%, 0.5%, 0.25%, and 0% to remove N2 from the medium. These cells were cultured in DFInsu (DF with 10 µg/ml human insulin) for a week, and then transferred to DF only. All media components were from GIBCO, Grand Island, NY.

To ensure that quantitative analysis of lineage restriction reflected a shift in phenotypic expression rather than changes in cell numbers, the cell lines were plated at equivalent concentrations (3×10^4 cells/well on 22-mm round glass coverslips). Double blind counts from triplicate experiments were made from randomized phase contrast micrographs at 4 DIV. Similarly, quantitative analysis of lineage restriction was completed by the determination of the percentage of each phenotype expressed in each of the different growth media at 4 DIV. Counts were made from randomized confocal micrographs.

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay

Cell viability for both lines was measured with a MTT kit (Sigma, St. Louis, MO). The increase of the MTT level corresponded to an increase in health cells (Carmichael et al., 1987). Cells were plated at a density of 5×10^4 cells/well in 24-well plates (Falcon, Franklin Lakes, NJ) and incubated at 37°C in a humidified atmosphere of 5% CO₂-95% air for 4 days. MTT solution, equal to 10% of the

medium was added to each well. Plates were incubated at 37°C for an additional 3 h; mitochondrial dehydrogenases in the living cells metabolized the MTT into a formazan product, which was measured by spectrophotometry at 570 nm.

Ornithine decarboxylase enzyme activity (ODC) assay

Polyamine synthesis is rate-controlled by the enzyme ornithine decarboxylase (ODC). An increase in its activity indicates an increase in synthetic activity. The NSFCs from each line (5×10^4 cells/well in 35-mm dishes, Corning) were seeded in DF, DFN2, DFB27, DFInsu, MEM10, DF 2.5 (DF with 2.5% FBS) or MEM2.5 (MEM with 2.5% FBS and 10 mg% gentamycin) to measure ODC activity (ODC kit Parkard Instrument Company, Inc., Downers Grove, IL; ^{14}C from Perkin Elmer life sciences, Inc., Boston, MA). Cells were harvested after 48 h and centrifuged at 10^4 rpm for 10 min at 4°C. Cell pellets were extracted with the TEP-L-DTT Buffer (300 μl of 50 mM Tris-HCl at pH 7.4, 0.1 mM EDTA, 10 mM dithiothreitol, 0.05 mM pyridoxal 5'-phosphate). Cells were lysed by three freeze-thaw cycles, and then centrifuged at 10^4 rpm for 10 min at 4°C. Supernatants were used immediately for the ODC assay and determination of protein concentration.

The ODC activity was measured as described earlier (Roisen et al., 1981a). Briefly, the assay mixture contained 100 μl cell extract and 10 μl [1-C^{14}] DL-ornithine hydrochloride (0.1 $\mu\text{Ci}/\mu\text{l}$). The assay was carried out in plastic tubes with two filter papers attached to the inside of the cap. The filter papers were moistened with 50 μl toluene-based scintillation fluid and solucence 350-tissue solubilizer to trap the released $^{14}\text{CO}_2$. After 1 h at 37°C with agitation, injection of 200 μl 10% tri-chloro-acetic acid through the cap stopped the reaction and the solution was incubated for an

additional 90 min. The incubation filters were removed and placed in 5 ml aqueous counting solution (ACS). Radioactivity was measured in a TRI-CARB 2100 TR liquid scintillation counter.

Protein concentration was determined using the Bicinchoninic acid (BCA) protein assay (procedures according to instructions of micro BCA protein assay reagent kit, Pierce Co., Rockford, IL).

Immunocytochemistry

Cells were plated at a density of 3×10^4 cells/well on 22 mm round glass coverslips in 6-well plates (Falcon) and incubated in a humidified atmosphere of 5% CO₂-95% air at 37°C for 4 days before they were fixed for immunofluorescence. Cultures were incubated with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (1:1000, 2 mg/ml, Molecular Probes, Eugene, OR) for 30 min at 37°C for vital labeling of DNA. The coverslips were rinsed with cytoskeletal buffer (CB: 2-*N*-Morpholino ethane sulfonic acid [MES] 1.95 mg/ml; NaCl, 8.76 mg/ml; 5 mM EGTA; 5 mM MgCl₂; glucose 0.9 mg/ml, pH 6.1) twice and fixed in 3% paraformaldehyde in CB for 10 min. Cells were treated with 0.2% Triton X-100 (Sigma) for 10 min for cytoplasmic antigens, and incubated in a blocking solution containing 3% bovine serum album (BSA) in Tris-Buffered Saline (TBS) for 1 h. For surface antigens, the Triton X-100 permeabilization step was omitted. Primary antibodies listed in Table 1 were applied at 4°C overnight. After washing in TBS three times for 1 h, the cells were incubated with the following secondary antibodies: Texas-red-conjugated goat anti-rabbit IgG, Cy2-conjugated goat anti-mouse IgG (all diluted 1:100, Cy2 from Jackson Immunology Research Laboratories, West Grove, PA; Texas red from

Molecular Probes). Omission controls were performed with each experiment to ensure the specificity of staining.

Statistical analysis

Statistical analysis (Graphpad Prism) was carried out using ANOVA; significance was set at the $P < 0.05$ level.

Table 1. Antibodies and specificity

Antibodies	Target	Source
Nestin, human monoclonal, 1:100	Neural stem cells	Chemicon International, Temecula, CA
Peripherin, polyclonal, 1:100	Neural crest cells	Chemicon
β -tubulin isotype III, monoclonal, 1:100	Neurons and progenitors	Sigma, St. Louis, MO
NCAM, human monoclonal, 1:100	Neurons	Chemicon
A2B5, monoclonal, 1:100	Glial precursors	Chemicon
GFAP, monoclonal and polyclonal, 1:100	Astrocytes	Chemicon
O4, monoclonal, 1:100	Oligodendrocyte precursors	Chemicon
GalC, polyclonal, 1:100	Oligodendrocyte precursors	Chemicon
RIP, monoclonal, 1:1000	Oligodendrocytes	Chemicon

RESULTS

NSFCs proliferated in DM in vitro

The NSFCs obtained from cultures of both living and postmortem tissue grow well in MEM with 10% serum, doubling approximately every 20 h. The oldest cultures have been passaged over 200 times. To evaluate the effect of growth factors and mitogens on the NSFCs, a totally defined medium was needed. Therefore, the NSFCs were weaned from MEM with 10% serum (Fig. 1A) by gradual reduction in serum concentration (Fig. 1B). Examination of the cells' reactions to two basic media, MEM and DF, revealed that MEM in the absence of serum could not support cell survival (data not shown) but DF could support cell survival and proliferation of NSFCs obtained from either postmortem or living donors.

However, NSFCs grown in DF alone required a high cell density (>400 cells/mm²), which resulted in a higher percentage of cells surviving to form neurospheres. After 2 days in vitro (DIV), these cells extended long very fine processes that were retracted by 4 DIV in DF. At this time, the cells detached from the substrate and round cells appeared (Fig. 1C). Following 12 DIV, these cells formed neurospheres. Thus, DF was selected for further study in combination with growth factors and supplements.

When NSFCs were cultured in DFN2, most of the cells formed long processes within 4 DIV (Fig. 1D). In addition, a small population of multinuclear giant cells also exhibited long processes (Fig. 1E). In long-term cultures (over 8 months), process formation decreased (Fig. 2A). When cells reached a high density, they retracted their processes, became round and formed neurospheres; in addition, with

time the number of giant cells increased. The change from MEM to DM, and plating on glass rather than on plastic influenced giant cell formation. These results were obtained irrespective of tissue source (postmortem or living donor).

Although giant cells appeared in the different culture conditions, they represented less than 2% of the total population. The giant cell population was heterogeneous and exhibited highly variable phenotypic expression. Approximately half of the giant cells expressed one or more neuronal markers. Frequently A2B5 reactivity was also noted. No keratin positive reactivity was observed. The giant cells appear to be a phenomenon of the culture milieu since the number of giant cells varied depending on the growth surface. Therefore, a detailed analysis was not undertaken.

When NSFCs were cultured in DFB27, cells formed long multiple processes (Fig. 1F). In cultures maintained for more than 4 months, the cells had a stellate morphology (Fig. 2B). These cells had a very short cell cycle and reached high density quickly after which, they formed numerous neurospheres.

The NSFCs, when transferred from DFN2 to DFInsu for a week followed by feeding with DF only, grew very slowly. In contrast, NSFCs maintained for over a week in DFInsu formed neurospheres (Fig. 2D). These cells grew slowly and developed bipolar morphologies with long processes for up to 6 months in vitro (Fig. 2E).

The NSFCs precultured in MEM 2.5 were evaluated for their ability to grow in a series of different media (Figs. 1A-F). Four of these media (DF, DFInsu, DFN2, and DFB27) supported long-term cell survival and proliferation (Figs. 2A-E). NSFCs

were maintained and expanded at present for 1 year in DFN2, DF, DFInsu, and DFB27, without the loss of mitogenic activity.

Cell viability

The NSFCs (96-year-old, passage 30-35) response to various media were analyzed by determination of cell viability using the MTT assay; an increase of the MTT value corresponded to increased cell number (Carmichael et al., 1987). Viability increased ($P < 0.01$) when NSFCs were grown in DFN2, DFB27, and MEM10 compared to DF (Fig. 3A). The growth factors in DF-brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF), ciliary neurotrophic factor (CNTF), epidermal growth factor (EGF), nerve growth factor (NGF) and neurotrophic factor 3 (NT-3)-had no detectable effect on cell viability (Fig. 3B).

Since N2 supplement had a major effect on NSFCs viability, the five components of N2 were tested individually. The individual components had less of an effect on cell viability than the complete N2 supplement (Fig. 3 and Fig. 4). To determine the dose response of NSFCs to the individual components of N2, NSFCs were cultured (4 DIV) in DF with a constant concentration for four of the five major components of N2 while the fifth component was varied. The concentration of putrescine, selenite, and transferrin at which the NSFCs exhibited a maximal growth response corresponded to the concentration found in N2. However, the concentration of insulin and progesterone that elicited the maximal response from the cells was greater than that found in N2 perhaps indicating a greater need for these two

components by the olfactory progenitors than other cell types. Furthermore, selenium was cytotoxic at concentrations of more than 3000 nm (Fig. 4).

ODC assay

To examine the effect of the media and supplements on polyamine synthesis after 2 DIV, NSFCs from the male cadaver-derived cell line (96-year-old, passage 45-50) were analyzed for ODC activity. All supplements except insulin increased synthetic activity while DF supplemented with B27 increased ODC activity many times over the levels obtained for the DF control ($P < 0.01$, Table 2). Similar results with other lines have been observed (data not shown).

Immunocytochemistry

The influence of the various media (MEM10, MEM2.5, DF, DF2.5, DFN2, and DFB27) on lineage restriction of the NSFCs was determined (Table 4). To evaluate changes in the percentage of phenotypic expression, cellular growth response to the various media was determined to ensure that any shift in lineage restriction was due to phenotypic expression rather than an increase in cell number. No differences in the cell number in cultures grown in MEM10, DF2.5, DFN2, and DFB27 were noted (Table 3). Thus, a detailed analysis of the effect of media on phenotypic expression was completed (Table 4).

In defined medium alone or supplemented with N2, over 97% NSFCs were positive for β -tubulin isotype III and peripherin (DF: Fig. 5A; DFN2: Fig. 5B). Peripherin usually was found surrounding the nuclei, but also was noted in some processes extending to the growth cones (Fig. 5C). The percentage of nestin positive cells decreased ($P < 0.01$) in cultures grown in DM, MEM2.5, and DF2.5 (Figs. 5D-F)

compared to those grown in MEM10 (Table 4). There was an increase in the percentage of A2B5 and NCAM positive cells ($P < 0.01$) in the cultures grown in DM (Figs. 6A-D). Under the various culture conditions, no keratin positive cells were observed within any of the subcultures. Among the cultures grown in MEM10, MEM2.5, DF, DFInsu, DF2.5, and DFN2, rare cells were observed positive for either MAP2, GFAP, RIP, GalC, or O4 (Table 4).

However, when cells were cultured in DFB27, MAP2 positive doughnut-like structures and short linear segments were present in more than 50% of the cells (Fig. 6F). After 3 months in DFB27, cells were found that were positive for O4 (<1%; immunohistochemistry not shown), galactosylceramide (GalC: 2%) and RIP (<1%). Cultures maintained in DFInsu for 6 months exhibited more A2B5 positive cells (91%; Fig. 6E) than in MEM10.

Figure 1. Since it was difficult to maintain viable cultures in MEM in the absence of serum, a defined culture medium was necessary. NSFCs from a 96-year-old male (passage 30-35) were used to evaluate the effects of alternative media. Representative micrographs illustrating the resultant phenotypic expression of NSFCs in (A) MEM10, (B) MEM2.5, (C) DF, (D) DF2.5, (E) DFN2, (F) DFB27. DF supplemented with either N2 or B27 resulted in robust mitotic activity, which was accompanied by extensive process formation within 4 DIV.

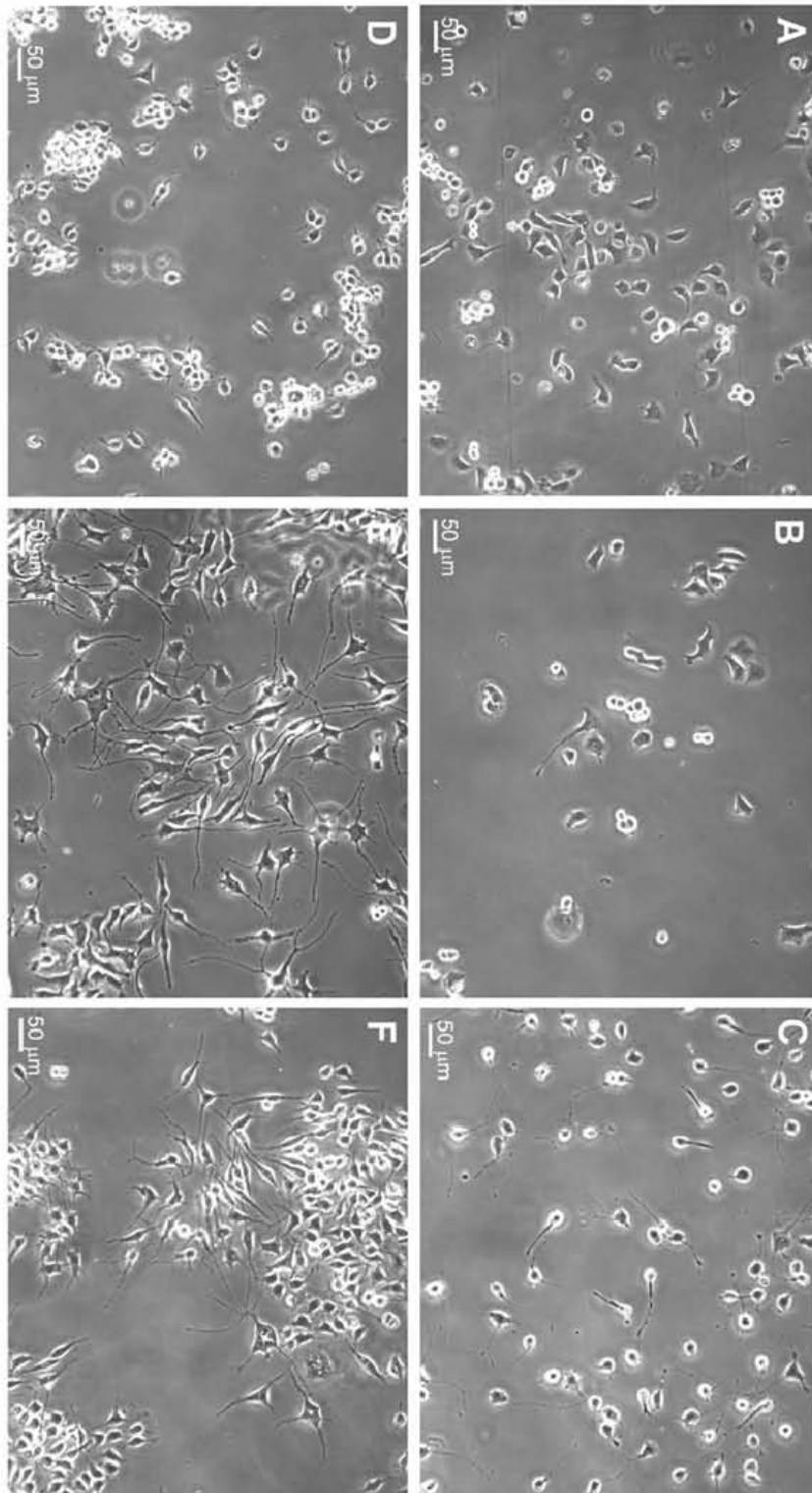


Figure 2. In the absence of serum, NSFCs from a 96-year-old male were maintained over prolonged periods in different media. (A) DFN2: 8 months IV (passage 73); (B) DFB27: 4 months IV (passage 67); (C) DF only: 6 months IV (passage 15); (D and E) DFInsu: 6 months (passage 31). B27-supplemented medium was evaluated for 4 months. This reflected the termination of the experiment rather than degeneration of the cells. The rate of mitotic activity varied depending on the medium. DF was passaged every 12 days, DFInsu every 6 days, DFN2 every 4 days. The mitotic activity supported by B27 required passage every 2 days.

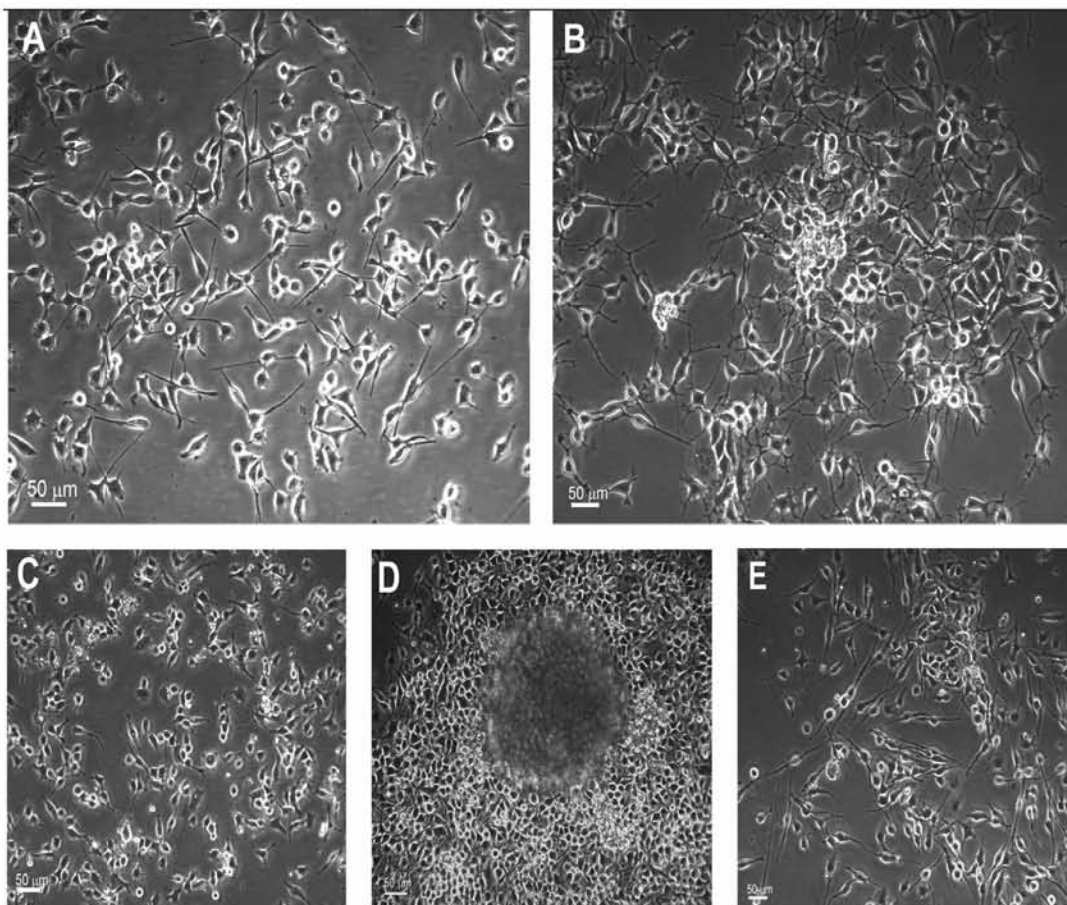


Figure 3. (A): NSFCs from a 96-year-old male (passage 30-35) were cultured in DF supplemented with various growth factors and assayed for level of viability using the MTT assay. After 4 DIV, cell viability increased significantly in medium supplemented with N2. The addition of serum and B27 supplement also increased viability of NSFCs. The supplement, B27® (GIBCO) produced the most pronounced effect, which was equivalent to the response obtained with MEM10. Experiments were performed in triplicate and averaged. DFi (DF + 5 µg/ml insulin), DFo (DF + 20 nM Progesterone), DFu (DF + 100 µM Putrescine), DFs (DF + 30 nM Selenite), DFt: (DF + 100 µg/ml transferrin). (B): After 4 DIV, NSFCs grown in DF supplemented with growth factors and N2 were analyzed. Only N2 showed a significant change in cell viability. The growth factors BDNF (20 ng/ml), bFGF (20 ng/ml), CNTF (50 ng/ml), EGF (10 ng/ml), NGF (20 ng/ml), and NT-3 (20 ng/ml) did not produce significant increases. Data are expressed as $X \pm \text{SEM}$ of each experiment of triplicate samples repeated a minimum of three times. * $P < 0.05$, ** $P < 0.01$.

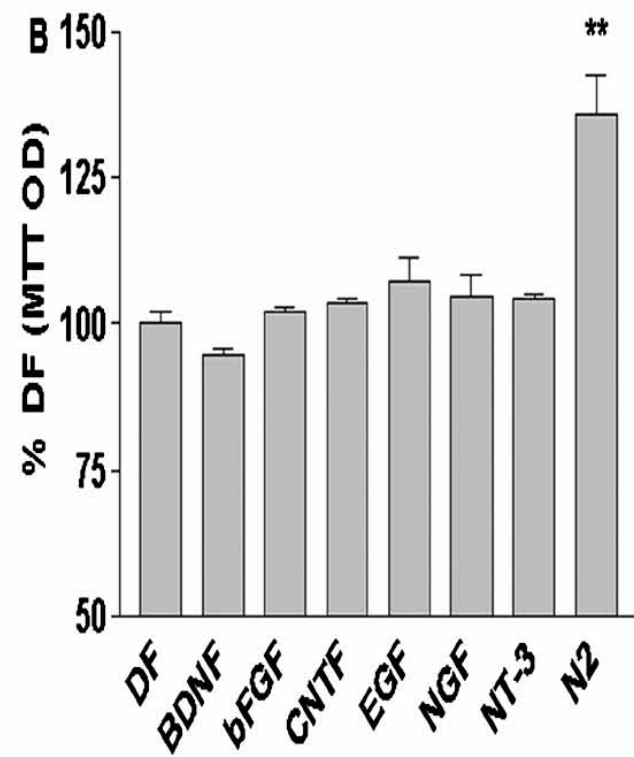
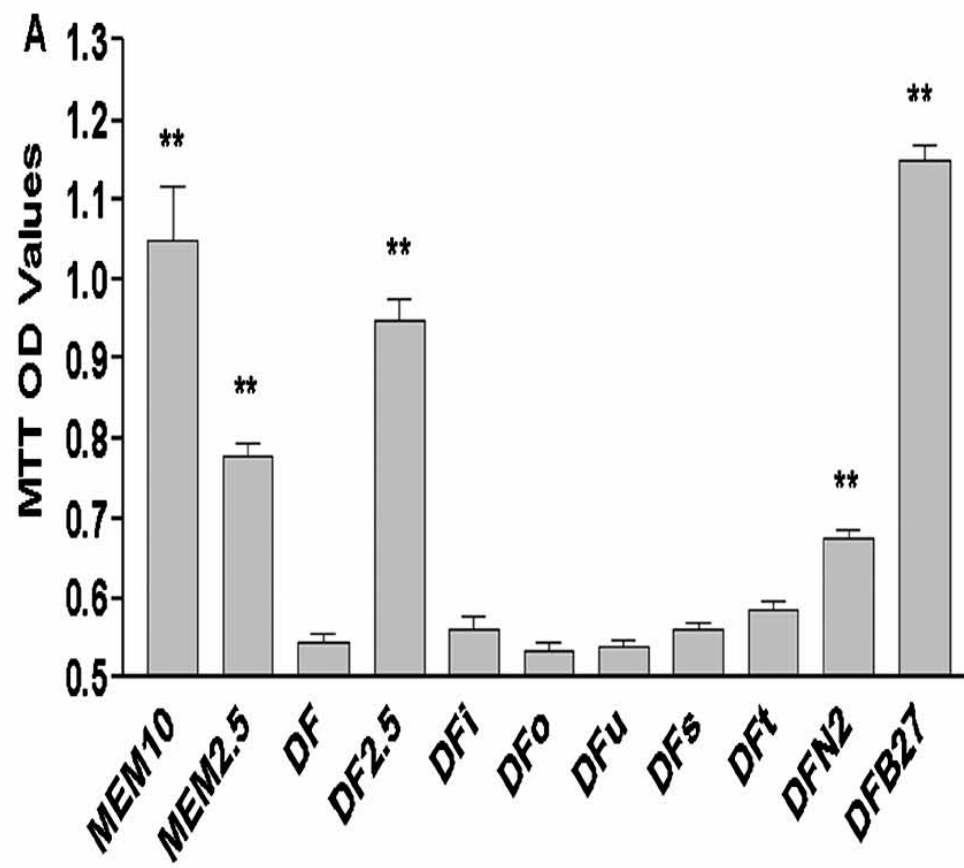


Figure 4. To determine the effect of each of the individual components of the N2 supplement, individual components were analyzed. NSFCs from a 96-year male (passage 40-50) were grown in DF with a constant concentration for four of the five components of N2 while the fifth component was varied for 4 DIV. The dose response of NSFCs to each of the individual components was determined. The maximal response of NSFCs occurred at the concentration present in N2 supplement except for two components, insulin and progesterone, perhaps reflecting the fact that human NSFCs may require higher levels of progesterone (20 nM) and insulin (5 µg/ml). After 4 DIV, no individual component was responsible for the increased viability. Data are expressed as $X \pm \text{SEM}$ of each experiment of triplicate samples repeated a minimum of three times. * $P < 0.05$, ** $P < 0.01$.

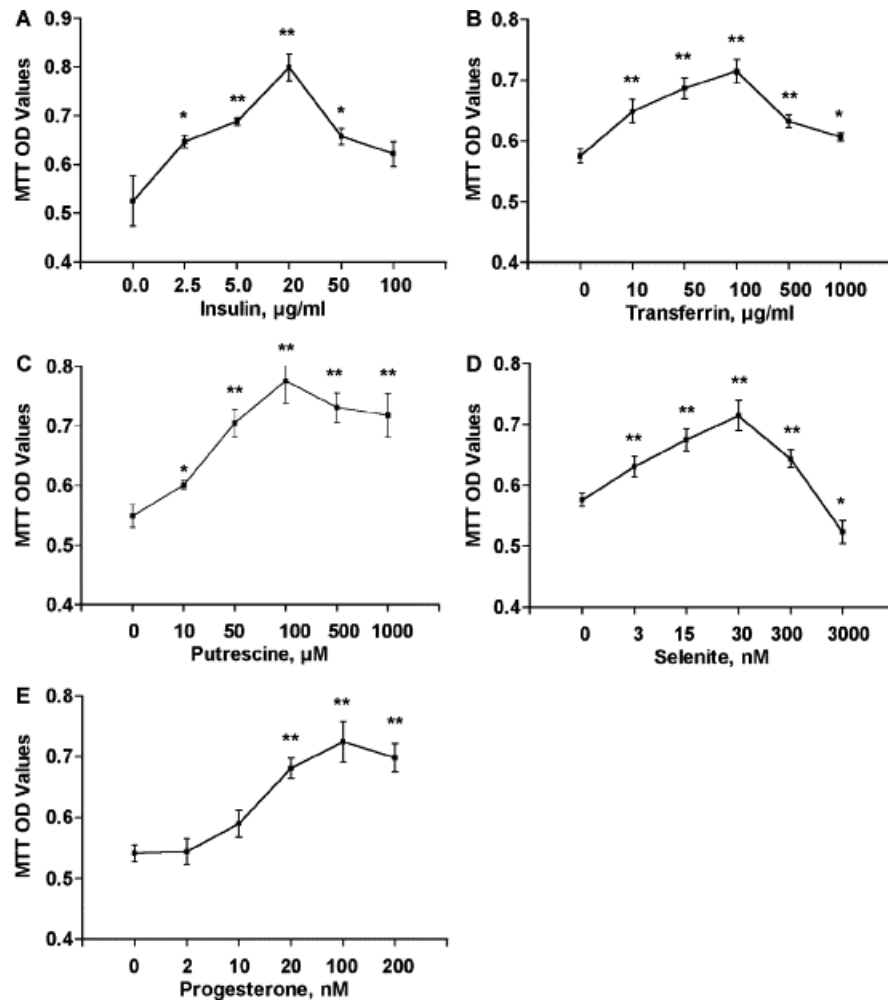


Table 2. Effect of media on ODC activity of NSFCs

Media	DPM/mg protein	% Control
MEM10	2900 \pm 110	740 **
MEM2.5	1000 \pm 50	260 **
DF (Control)	390 \pm 40	100
DF2.5	6500 \pm 310	1700 **
DFInsu	500 \pm 60	130
DFN2	1800 \pm 130	460 **
DFB27	18000 \pm 1500	4600 **

No variation was observed between the cell lines studied.

Table 3. Effect of media on the number of NSFCs/coverlip (4 DIV)

Media	Cells/mm ²	% Control
MEM10	222 ± 19	225 **
MEM2.5	113 ± 4	114
DF (Control)	99 ± 19	100
DF2.5	245 ± 42	247 **
DFN2	212 ± 41	214 **
DFB27	291 ± 34	294 **

Analysis of cell density obtained for the groups presented in Table 4. No variation was observed between the cell lines.

Table 4. Effect of media on the lineage restriction of NSFCs (4 DIV)

Antigen	Percentage of positive cells								
n	MEM1 0(4 DIV)	MEM2 .5(4 DIV)	DF(4 DIV)	DF2. 5(4 DIV)	DFN 2(4 DIV)	DFB 27(4 DIV)	DFB27(3month)	DFIns u(4 DIV)	DFInsu(6month)
β-tubulin	>97	>97	>97	>97	>97	92.8 ±8.2	91.3±6.9	>97	>97
III									
Peripherin	>97	>97	>97	>97	>97	>97	>97	>97	>97
Nestin	78.2±2.5	39.2±2.4**	68.8 ±7.6	55.4 ±6.3**	36.2 ±3.8**	48.0 ±3.4**	46.9±5.3**	45.5±3.6**	45.0±1.2**
A2B5	13.5±3.6	61.8±4.9**	75.8 ±4.6**	40.2 ±3.3	92.5 ±2.5**	77.3 ±6.8**	83.3±3.1**	79.7±5.9**	90.5±4.5**
NCAM	20.0±2.0	29.1±4.9	35.5 ±2.9*	25.7 ±3.2	87.0 ±1.5**	43.2 ±1.8**	63.8±7.4**	81.0±9.6**	89.3±3.2**
O4	0	0	0	0	0	0		NA	0
GALC	0	0	0	0	0	0		NA	0
RIP	0	0	0	0	0	0		NA	0
GFAP	<1	0	0	0	0	0	0	NA	0
MAP2	<1	0	0	0	0	0	50.8±2.8	NA	11.3±1.3
							a		a

Quantitative analysis of immunofluorescent localization demonstrated the expression of different phenotypes in response to changes in serum concentration and with the addition of supplements compared to control (MEM10). All treatments resulted in a decrease in nestin expression except for DF, perhaps reflecting differentiation of early progenitor (stem) cells toward glial (A2B5) or neuronal (NCAM) lineages. NCAM expression by the NSFCs increased in response to DF plus N2 or B27. A2B5 expression increased as the serum level decreased.

a MAP2 positive doughnut-like structures and short linear segments.

*P < 0.05 compared to MEM10 control.

**P < 0.01 compared to MEM10 control.

Figure 5. Representative micrographs of NSFCs obtained from a 22 year old female via endoscopic biopsy, Passage 11 (A, D, E) and from 96 year old male cadaver, passage 40-50 (B, C, F), 4 DIV. More than 97% cells were positive for β -tubulin isotype III and peripherin in (A) DF and (B, C) DFN2. A small but significant population of nestin positive cells was constantly found in all NSFC subcultures, (D) MEM10; (E) DF; and (F) DFB27. Immunolocalization as indicated in plate. DAPI vital staining was employed to demonstrate nuclei. (Confocal Microscopy)

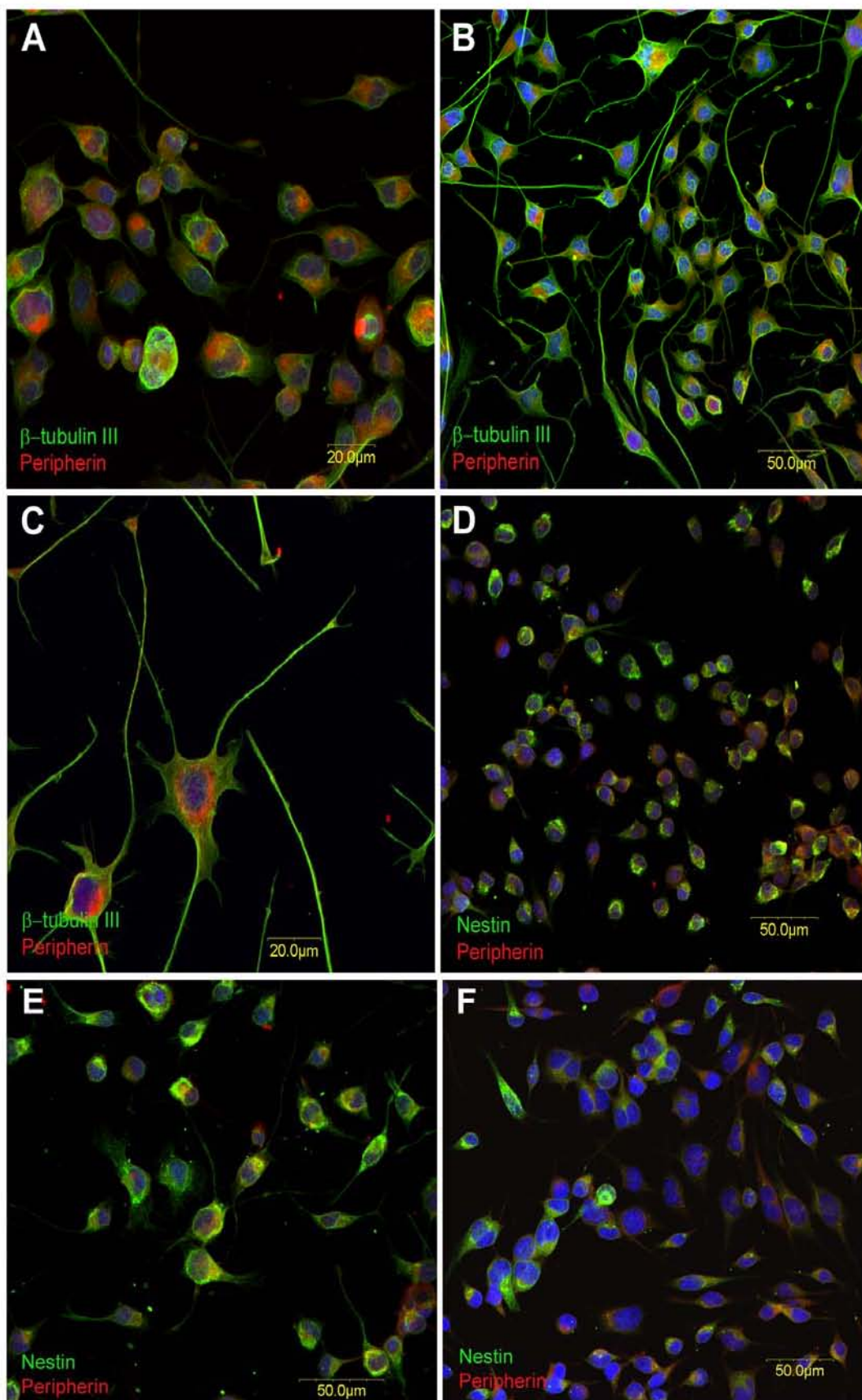
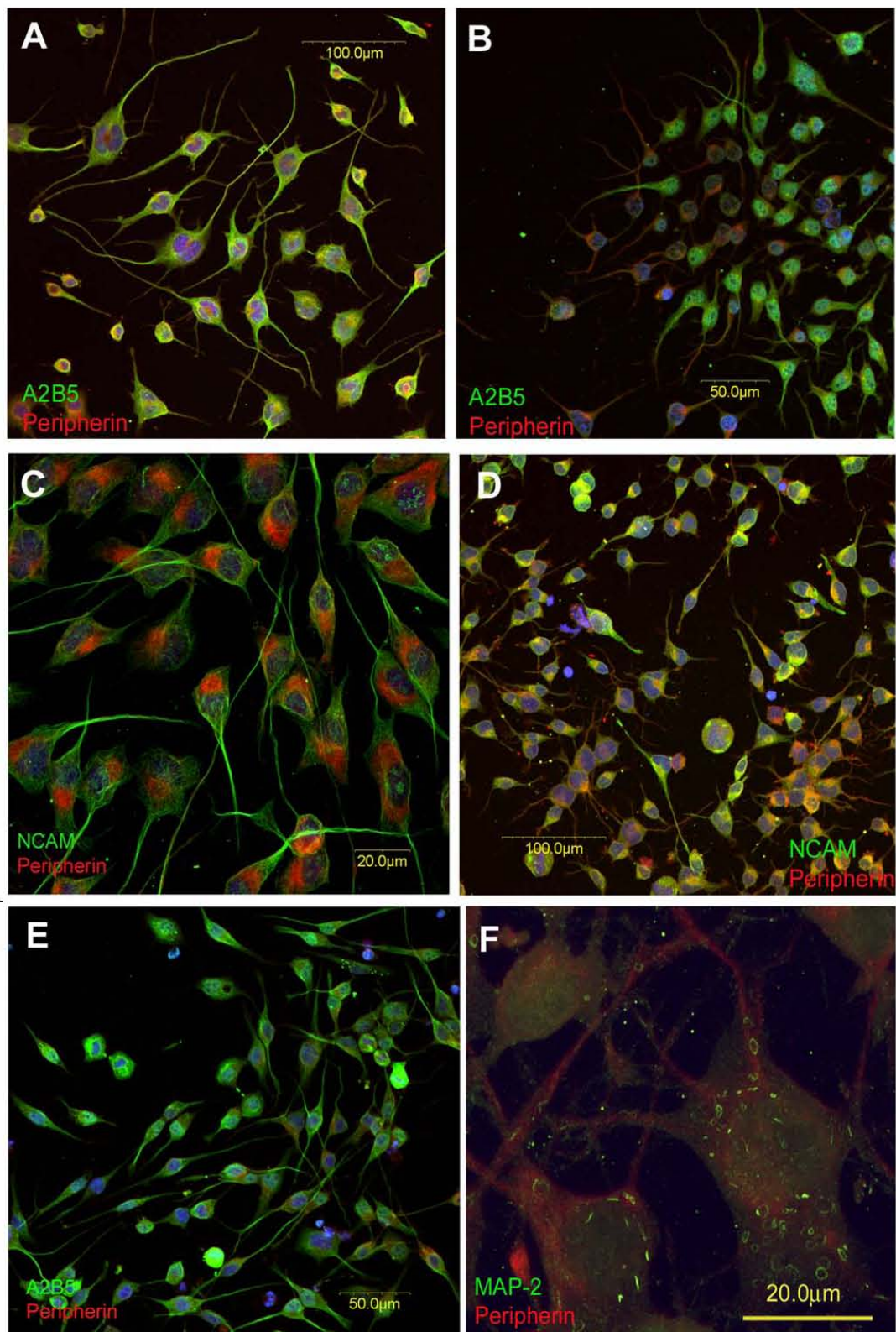


Figure 6. Representative micrographs of NSFCs obtained from 96-year-old male cadaver, passage 40-55. A2B5 and NCAM positive cells were increased in DM, (A, C) DFN2; (B, D) DFB27, 4 DIV. (E) More A2B5 positive cells were found in DFInsu after 6 months of culture than in MEM. (F) MAP2 positive doughnut-like structures and short linear segments in more than 50% cells maintained in DFB27 in 3 months. (Confocal Microscopy)



DISCUSSION

The NSFCs used in this study were derived from adult OE and have been characterized as adult human progenitors (Roisen et al., 2001). The purpose of the current study was to further define and characterize these cells. The original medium used to establish the NSFC lines was highly enriched with 10% serum (Roisen et al., 2001). The composition of serum is not fully known, and it may contain bioactive materials that might mask the effects of exogenous growth factors (Hashimoto et al., 2000). Thus, a defined medium would enable precise culture conditions and permit examination of the effects of exogenous growth factors on the growth and differentiation of these adult human stem cells.

In the present study, the adult NSFCs consisted of heterogeneous populations in which some cells exhibited immunoreactivity to nestin, NCAM, A2B5, β -tubulin III and/or peripherin when cultured in DM. The diversity of these markers suggests that OE cells are pluripotent with an ability to differentiate along either glial or neuronal lineage. Nestin reactivity has been associated with neural stem cells (Espinosa-Jeffrey et al., 2002) including those derived from embryonic (Espinosa-Jeffrey et al., 2002), neonatal (Zhang et al., 1998) and adult rodent CNS (Gritti et al., 1996) as well as embryonic human brain (Tohyama et al., 1992). Nestin positive cells have been shown to differentiate into neurons, astrocytes, and oligodendrocytes when mitogens are withdrawn or they are exposed to other factors (Gage, 2000; McKay, 1997; Svendsen et al., 1996). Stem cells restricted along a neuronal lineage have been described as immunoreactive for NCAM, β -tubulin III and peripherin (Roisen et al., 2001). Peripherin is an intermediate-filament found in cells derived from the neural

crest (Djabali et al., 1991). Since cranial nerves are part of the peripheral nervous system and are derived from neural crest, it was not surprising to find peripherin in the adult OE-derived NSFCs. Embryonic rat neuroepithelium (Rao and Mayer-Proschel, 1997) and fetal human brain (Dietrich et al., 2002) derived stem cells immunopositive for A2B5 were shown to differentiate into astrocytes and oligodendrocytes, but failed to develop into neurons when exposed to platelet-derived growth factor (PDGF), CNTF, and T3 (Dietrich et al., 2002; Rao and Mayer-Proschel, 1997).

The human NSFCs cultured in MEM10 remain mitotically active and form neurospheres (Roisen et al., 2001). When the medium was changed to MEM without serum, the cells did not survive. However, if the cells were slowly weaned from serum in DM, NSFCs survived and proliferated. Cell viability and ODC activity were used to measure the cells' responses to changes in media. ODC, the rate-limiting enzyme in the polyamine biosynthetic pathway, has been shown to play an important role in the regulation of cell growth, proliferation, and differentiation (Evangelisti et al., 1999; Gupta et al., 1999; Marton and Pegg, 1995; Nilsson et al., 2000). The concentration of the polyamines is highest in S phase during the cell cycle because it is needed for DNA synthesis (Evangelisti et al., 1999).

When the five major components of N2 were applied individually, none of the components appeared to have the effect of N2 itself on either line. In DF and DFInsu, cell viability and ODC decreased suggesting that DF and DFInsu only minimally supported the nutritional needs of the NSFCs. It was also found that increased plating density resulted in a higher percentage of cells surviving to form neurospheres in DM,

especially in DF. When cells from either line were cultured in DF in the presence of N2 supplement, there was an increase in cell viability and ODC activity. Thus, NSFCs required all of the components in N2 for survival. However, there were two components (insulin and progesterone) that the adult olfactory NSFCs required at a higher concentration than was present in N2 for an optimal response that appears different from other cells. For example, B104 cells exhibited a maximal growth response to the concentration of insulin and progesterone found in N2 (Bottenstein and Sato, 1979). However, oligodendrocyte progenitors derived from neonatal mice CNS needed higher concentrations of insulin and progesterone for their survival in vitro than that found in N2 (Vitry et al., 1999). Insulin is very similar to insulin-like growth factor (IGF), which is required for neuronal survival and differentiation (Mackay-Sim and Chuah, 2000). Many components of the insulin/IGF signaling system are found in olfactory epithelium (Mackay-Sim and Chuah, 2000). Therefore, the need for higher levels of insulin and progesterone likely reflects the unique requirements of adult OE progenitors.

The supplement, B27, outperformed N2 as indicated by the increased MTT levels and ODC activity of NSFCs from both lines. The composition of B27 includes many hormones, anti-oxidants and retinal acetate in addition to the five components of N2. However, the concentration of each component in B27 is proprietary information of GIBCO and was not available. When NSFCs were grown in B27 compared to B27 without anti-oxidants no growth differences were noted (data not shown). It is not known which factors in B27 were responsible for the increases in MTT and ODC activity. It may be the result of the combination of all these factors as

shown by Svendsen et al. (1995). None of the B27 components (retinal acetate, tocopherol, catalase, and super oxide dismutase) in EGF-supplemented N2 medium had the same effect as B27 alone on CNS precursor cells obtained from embryonic rat mesencephalon and striatum (Svendsen et al., 1995).

In addition to the metabolic changes induced by the media, the NSFCs from both lines exhibited similar morphologic changes. In MEM10, most of NSFCs were nestin, β -tubulin III and peripherin positive. Cells positive for A2B5 and NCAM made up less than 25% of the population; only a few cells exhibited MAP2 or GFAP reactivity (Roisen et al., 2001). Nestin is an intermediate filament protein expressed by a variety of undifferentiated pluripotent neuroectodermal precursor cells (Espinosa-Jeffrey et al., 2002; Gritti et al., 1996; Tohyama et al., 1992; Zhang et al., 1998). In contrast, A2B5 is expressed by primarily glial precursors (Dietrich et al., 2002; Rao and Mayer-Proschel, 1997); β -tubulin III and NCAM are expressed in immature and mature neurons (Mackay-Sim and Chuah, 2000; Rao and Mayer-Proschel, 1997; Roisen et al., 2001). Thus, this immunohistochemical profile implies that the NSFCs grown in MEM10 were primarily undifferentiated neuronal progenitors. With the withdrawal of serum, the population of nestin positive cells decreased while the population of A2B5 and NCAM positive cells increased. The presence of NCAM and β -tubulin III positive cells suggests that some of the cells had begun to follow lineage-restricted paths and were differentiating toward a neuronal lineage. Cells expressing neuronal markers were observed more frequently in DM than cells expressing glial markers. Thus, the differentiation potential of human progenitor cells was restricted (Fricker et al., 1999; Quinn et al., 1999).

A subset of the A2B5 positive cells from either line coexpressed nestin and neuronal markers such as NCAM or β -tubulin III. Few cells expressed MAP2 (neuron) or GFAP (glia) markers characteristic of more mature differentiated phenotypes. Cells, which coexpressed NCAM and A2B5, were abundant in cultures grown in DFN2 and DFB27. This observation is not unique to NSFC progenitor cells and is similar to observations on other cell lines. Co-expression of lineage-restricted markers has been noted in human embryonic stem cells (Carpenter et al., 2001; Dietrich et al., 2002), rat embryonic stem cells from the cortex and neural tube (Espinosa-Jeffrey et al., 2002; Rao and Mayer-Proschel, 1997), and adult progenitors from the rat hippocampus (Gage et al., 1995). Coexpression of lineage-restricted markers may indicate a transition phase in the development of lineage restriction. Thus, it appears that adult-derived OE NSFCs progenitors have not made a final commitment. Cells, which expressed astrocytic markers such as GFAP and mature neuronal markers such as MAP2, were not identified. However, after 3 months in DFB27, more than 50% of the cells had MAP2 reactive positive structures in the cytoplasm suggesting that a subpopulation of NSFCs was beginning to differentiate into more mature neurons. In addition, a few A2B5 positive cells appear to be differentiating into mature oligodendrocytes, as indicated by immunoreactivity for O4, GalC, and RIP.

Under the defined experimental conditions, none of the neurotrophic factors (NTFs) evaluated increased NSFCs viability or lineage restriction. This result is different from that reported concerning other adult neural progenitors. The NSFCs derived from bFGF-responsive neuronal precursors of the mouse postnatal dorsal root

ganglia were induced to differentiate into neurons after removal of bFGF and addition of trophic factors (Namaka et al., 2001). Adult neural stem cells reside in the CNS as quiescent cells and they need NTFs to proliferate (Fricker et al., 1999; Gage, 2000). The olfactory receptors are directly exposed to the environment and may easily be injured. The OE NSFCs differ from other stem cells because throughout life they generate new cells to repopulate the OE every 30-40 days (Caggiano et al., 1994; Graziadei and Monti-Graziadei, 1979; Moulton, 1974). Thus, NTFs may not play as pivotal a role in their mitotic activity as other stem cells.

A small population of giant cells was found in the cultures obtained from either the postmortem or living donors when NSFCs were grown in DM, on glass rather than plastic or grown at high density. The giant cell population may reflect either incomplete cell cytokinesis or alternatively fusion of existing cells. These cells do not appear to be related to the giant cells that are frequently found in the primary OE cultures (Liu et al., 1998; Othman et al., 2003), since they did not have a well-defined keratin positive filamentous network. Instead, these cells are likely to be a phenomenon of the culture milieu since their numbers depended on the growth surface. Similar results were noted by Couso et al. (2002). In vitro multinucleated giant cells (MGS) arose from mononuclear macrophages due to cell fusion when plated at high density. This formation of MGS was considered as part of specialized macrophage differentiation. Kaplan and Gaudernack (1982) also showed that human macrophages cultured on glass surfaces formed MGS, but not when the cells were grown on collagen matrices. In all cultures grown in DM, some cell aggregation occurred immediately after plating. A possible reason for this phenomenon is that the

cells require cell-cell contact in these aggregates to support further cell proliferation. They may produce growth-promoting factors, which act in a paracrine and/or autocrine manner similar to that noted by Gao et al. (1991). A similar relationship in the OE cultures related to BDNF has been noted (unpublished data). In vitro, precursor cells from early postnatal mouse cerebellum exhibited increased DNA synthesis when they reaggregated compared to those dispersed in culture (Gao et al., 1991).

In summary, irrespective of the tissue source (postmortem or living donor):

1. MEM in the absence of serum did not support NSFC survival;
2. DF after weaning supported NSFC survival and proliferation;
3. MTT and ODC levels increased when NSFCs were maintained in DM supplemented with either N2 or B27;
4. Although N2 supplement increased the viability of NSFCs in the absence of serum, no one individual component of N2 was responsible for the effect;
5. Under the defined experimental conditions, none of the neurotrophic factors evaluated increased NSFCs viability or lineage restriction; and
6. Phenotypic expression of the NSFCs could be altered by growth in different media.

In general, nestin reactivity decreased as serum concentration decreased. A2B5 reactivity increased in the absence of serum while NCAM reactivity was greatly increased by N2 and B27 supplements.

Adult human olfactory derived progenitors provide a unique cell model for evaluating factors required for their lineage restriction in vitro. Furthermore, these studies demonstrated that long-term cultures of NSFCs from either living or

postmortem olfactory neuroepithelium can be maintained in defined media without loss of mitotic potential. Modulation of these cells in vitro may develop procedures for obtaining novel populations of progenitors for transplantation into diseased or damaged CNS or for use in drug discovery and pharmacological evaluation. Furthermore, the location of the olfactory epithelium would allow that cells derived from the patient could be used for autografts thus insuring complete graft-host compatibility and eliminating the need for immunosuppressive therapy. The unique potential of these cells warrants further study.

ACKNOWLEDGEMENTS

This study was supported by NIH Grant 1920RR15576. The authors are grateful to Dr. Linda M. Hatcher for assistance in manuscript preparation.

CHAPTER III

INDUCTION OF OLIGODENDROCYTES FROM ADULT HUMAN

OLFACTORY EPITHELIAL-DERIVED PROGENITORS BY

TRANSCRIPTION FACTORS

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Published in Stem Cells 2005, 23 (3): 442-453.

INTRODUCTION

In the olfactory neuroepithelium, the receptor neurons and their supporting cells arise from a population of basal stem cells, which are responsible for their lifelong replacement (Moulton, 1974; Graziadei and Graziadei, 1979; Calof and Chikaraishi, 1989; Zhang et al., 2000). Dissociated cultures of adult olfactory neuroepithelium isolated from cadavers (Roisen et al., 2001), or patients undergoing endoscopic nasal sinus surgery (Winstead et al., 2005), produced neurosphere-forming cells (NSFCs) that have been used to generate approximately 60 lines. These lines may have the potential to differentiate into neurons or glia depending on their environmental signals. Thus, they may have the potential to be used therapeutically to treat neurological disorders such as the demyelinating diseases in which oligodendrocytes are selectively lost (Duncan et al., 1997; Scherer, 1997).

Oligodendrocytes are macroglial cells that form myelin in the central nervous system (CNS), as well as modulate the activities of adjacent neurons by regulating their microenvironment (Ludwin, 1997). The mechanisms underlying oligodendrocytic specification and differentiation from embryonic neural stem or progenitor cells are under extensive investigation. During development, oligodendrocytes arise from restricted loci of neuroepithelial precursor cells in the ventral neural tube (Warf et al., 1997; Noll and Miller, 1993; Ono et al., 1995; Orentas et al., 1999; Richardson et al., 2000) under the influence of the ventral midline signal sonic hedgehog (Roelink et al., 1995; Trousse et al., 1995; Poncet et al., 1996; Pringle et al., 1996). In the early stage of oligodendrogenesis, the basic helix-

loop-helix transcription factors *Olig1* and *Olig2* are initially expressed in oligodendrocyte-generative zones of the neuroepithelium. As oligodendrocyte progenitors leave the ventricular zone, *Olig1/2* expression is retained in oligodendrocyte progenitors and persists in mature oligodendrocytes (Zhou et al., 2000; Lu et al., 2000; Qi et al., 2001). Molecular and genetic studies have demonstrated that expression of the *Olig* genes is required for oligodendrocyte lineage determination in vivo (Lu et al., 2002; Zhou et al., 2002, Takebayashi et al., 2002). Interestingly, either before or after oligodendrocyte progenitors migrate into the white matter, they acquire the expression of two other transcription factors, the high-mobility transcriptional regulator *Sox10* (Stolt et al., 2002) and the homeodomain transcription factor *Nkx2.2* (Xu et al., 2000; Fu et al., 2001; Zhou et al., 2001; Fu et al.; 2002). The expression of *Nkx2.2* and *Sox10* seems to directly regulate myelin gene expression and oligodendrocyte differentiation; mutations of both genes result in a decreased number of mature oligodendrocytes in the CNS (Qi et al., 2001, Stolt et al., 2002). Conversely, expression of *Nkx2.2* in combination with *Olig2* can induce ectopic formation of mature myelin basic protein (MBP)-positive oligodendrocytes in embryonic chicken spinal cord (Zhou et al., 2001).

The role of these transcription factors in the differentiation of glial cells from human-adult derived neural stem cells has not been demonstrated. Thus, the purpose of this study was to investigate the roles of *Olig2* and *Nkx2.2* genes in human oligodendrocyte lineage specification and differentiation in vitro using adult human olfactory neuroepithelial progenitors. In this study we report that the simultaneous

transfection of NSFCs with *Olig2* and *Nkx2.2* or *Sox10* and *Nkx2.2* can lead to oligodendroglial morphology and lineage-restricted marker expression.

MATERIALS AND METHODS

Cell culture

The two different NSFC lines used in this study were obtained from adult olfactory neuroepithelium, one from a cadaver of a 96-year-old man (Roisen et al., 2001) and the other from a 22-year-old female patient via endoscopic biopsy (Winstead et al., 2005). Procedures for the harvest of these lines from primary cultures have been previously described (Roisen et al., 2001; Winstead et al., 2005). Frozen stock (passage 3 through 6) of each cell line was thawed rapidly, and 5×10^5 cells were placed in each flask in minimal essential medium (MEM) with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO, Grand Island, NY), 10 mg/100ml gentamycin (MEM10) in flasks (25 cm², Corning Incorporated, Corning, NY) in humidified 5% CO₂/95% air (37°C) for 24 hours. The NSFCs were adapted to the absence of serum via serial dilution of serum every 2 days for 1 week until the cells were finally cultured in DFB27M (DMEM/F12 supplemented with 2% B27) and 10 mg/100ml gentamycin for 1 week (Zhang et al., 2004) and then used for in vitro differentiation analyses between passages 10 and 20. Parallel experiments were preformed on NSFCs with both lines to determine if patient-specific differences were obtained. Because equivalent results were obtained with these two different lines, data from only one line has been presented.

Construction of expression vectors

Full-length mouse *Olig2* cDNA was cloned into the pIRES2-enhanced green

fluorescent protein (EGFP) expression vector (Clontech, Palo Alto, CA). The *Nkx2.2* gene was isolated by screening the mouse 129Sv cDNA library and cloned into the pIRES2-EGFP expression vector. For the *Olig2* and *Nkx2.2* coexpression vector, *Olig2* cDNA was cloned into pIRES (Clontech) between NheI and EcoRI, and *Nkx2.2* cDNA was inserted between XbaI and SalI. Similarly, the chicken *Sox10* cDNA was cloned into the pIRES2-EGFP expression vector. For coexpression of *Sox10* and *Nkx2.2*, the chicken *Sox10* cDNA and mouse *Nkx2.2* were sequentially cloned into the pIRES expression vector. The pIRES2-EGFP and pIRES expression vectors served as controls. All expression vectors were verified by extensive DNA sequencing. A further control was provided by lipofectamine alone.

Transfection and selection

All plasmid constructs were introduced into the NSFCs by liposomal transfection. The cells were plated on glass coverslips in six-well plates (3×10^4 cells/35-mm well) in DFB27M without antibiotics 1 day before transfection. NSFCs were transfected with each plasmid (4 μ g/well) for 48 hours according to the manufacturer's protocol (Life Technologies, Rockville, MD). Two days after transfection, the cells were fixed or fed with DFB27M supplemented with G418 (50 μ g/ml; GIBCO, Grand Island, NY) for selection.

Cell process formation

NSFCs were plated on glass coverslips in six-well plates (3×10^4 cells/35-mm well) in DFB27M without antibiotics, transfected with each plasmid (4 μ g/well) for 48 hours, and selected by G418 for 7 days. Cells were seeded on coverslips in six-well plates (1×10^3 cells/35-mm well) in DFB27M without transfection and selection

for controls. On 1, 3, 5, 7, and 9 days in vitro (DIV) during transfection and selection, the number and length of processes were measured with the aid of an eyepiece retical under constant magnification with phase-contrast optics. Cells (500 to 1,000) were sampled systematically from standardized fields (total magnification, x 200). Only primary processes originating directly from the soma that were longer than the diameter of the cell body were evaluated. The cultures were coded before evaluation.

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay

The viability of the NSFCs after 2 days of transfection and 7 days of selection in flasks (25 cm², Corning Incorporated) was measured with MTT kit (Sigma). Cells were seeded in the flasks in DFB27M without transfection and selection as controls. Cells were plated at a density of 5×10^4 cells per well in 24-well plates (Falcon). MTT solution was added to each well. Mitochondrial dehydrogenases in living cells metabolized MTT into formazan crystals, the concentration of which was determined spectrophotometrically at a wavelength of 570 nm, as described previously (Zhang et al., 2004).

Immunocytochemistry

The NSFCs (3×10^4 cells/well) were plated on 22-mm round glass coverslips in six-well plates (Falcon, Franklin Lakes, NJ) and incubated at 37°C in 5% CO₂/95% air for 24 hours, transfected for 2 days, and either immediately fixed or selected for 1, 4, or 7 days before fixation for immunofluorescence. Cultures were incubated with 4',6-diamidino-2-phenylindole dihydrochloride (1:1,000, 2 mg/ml, Molecular Probes, Eugene, OR) for 30 minutes at 37°C for vital labeling of DNA when nuclear staining was desired. The coverslips were rinsed with cytoskeletal buffer (CB) (1.95 mg/ml 2-

N-Morpholino ethane sulfonic acid, 8.76 mg/ml NaCl, 5 mM EGTA, 5 mM MgCl₂, 0.9 mg/ml glucose; pH 6.1) twice and fixed in 3% paraformaldehyde in CB (10 minutes) when permeabilization was desired, treated with 0.2% Triton X-100 (Sigma) for 10 minutes, and incubated (1 hour) in 3% bovine serum albumin in Tris-buffered saline (TBS). Primary antibodies (Table 5) were applied overnight at 4°C. After washing (1 hour) in TBS three times, the cells were incubated with the following secondary antibodies: Texas red–conjugated goat anti-rabbit immunoglobulin G (IgG), Texas red–conjugated goat anti-mouse IgG, and Cy2-conjugated goat anti-mouse IgG (all diluted 1:100, Cy2, Jackson Immunology Research Laboratories, West Grove, PA; Texas red, Molecular Probes, Eugene, OR). Experiments were performed in triplicate; first and second antibody omission controls were performed with each experiment to ensure the specificity of staining.

Western blot analysis

Western blot analysis was used to support the immunofluorescence studies. Proteins from human fibroblasts (ATCC, CRL-1836), NSFCs cultured in DFB27M without selection, NSFCs transfected with control vectors, and NSFCs transfected with the vectors plus each combination of transcription factors (*Olig2*, *Nkx2.2*, *Olig2-Nkx2.2*, *Sox10*, and *Sox10-Nkx2.2*) were collected in lysis buffer (20 mM Tris-HCl containing 1% NP40, 10% glycerol, 137 mM NaCl, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 4 mM phenylmethylsulfonyl fluoride, 20 mM sodium fluoride (NaF), 1 mM Na₃VO₄, and 1% Triton X-100). After 10 minutes of incubation at 4°C, samples were centrifuged at 12,000g (20 minutes) and the protein concentration of the supernatant was determined. The protein samples (20 µg/well) were electrophoresed

on 10% to 14% SDS-polyacrylamide gels along with standardized molecular size marker proteins in an adjacent lane (150 V, 60 to 90 minutes) and transferred (100 V, 60 to 90 minutes) from gel to nitrocellulose paper. Nonspecific binding was blocked (1 hour) with 5% nonfat dry milk in TBST buffer (5 mM Tris-HCl [pH 7.6], 136 mM NaCl, 0.1% Tween 20). Blots were incubated (4°C overnight) after the sequential addition of each of the following primary antibodies: polyclonal anti-Olig2 (1:2,000 dilution), monoclonal anti-Nkx2.2 (1:50 dilution), polyclonal anti-human MBP (1:1,000 dilution), polyclonal anti-Sox10 (1:1,000 dilution), and monoclonal anti-actin (1:2,000 dilution, Amersham Life Science). Blots were washed with TBST buffer four times for 20 minutes; then three times for 10 minutes. Washed blots were incubated for 1 hour in polyclonal horseradish peroxidase-labeled anti-rabbit IgG (1:1,000) for Olig2, human MBP, and Sox10 as well as monoclonal horseradish peroxidase-labeled anti-mouse IgG (1:1,000) for Nkx2.2 and actin.

Chemiluminescence Western blotting detection (Bio-Rad, Hercules, CA) was used to identify bound antibodies. Densitometry of the protein bands was carried out on a Molecular Dynamics gel scanner (Molecular Dynamics, Sunnyvale, CA). Data were analyzed using the Image Quant software programs supplied by the manufacturer.

Coculture

Purified cultures of either EGFP mouse (Jackson Laboratory, Bar Harbor, ME) or rat dorsal root ganglia neurons (DRGNs) were established by classic techniques (Kleitman et al., 1991). Briefly, DRGNs from 1- to 3-day-old (P1 to P3) GFP mice or Sprague-Dawley rats were purified and maintained in MEM10 for 4 DIV on glass coverslips in six-well plates; then in DFB27M for 3 weeks before seeding of NSFCs.

The medium was supplemented with nerve growth factor (NGF) (50 ng/ml; Sigma) and dibutyryl cyclic AMP (0.5 mM; Sigma), which stimulated axonal elongation (Roisen et al., 1972a; 1972b). On 2 to 4, 6 to 8, and 10 to 12 DIV, fluorodeoxyuridine (Sigma) was added to the media to reduce the nonneuronal (dividing) cells.

NSFCs for coculture with green fluorescent protein (GFP) mouse DRGNs or retroviral-labeled with GFP NSFCs for coculture with rat DRGNs after transfection with *Olig2-Nkx2.2* or *Sox10-Nkx2.2* and selection were maintained in DFB27M for 1 day and then detached to seed onto established DRGN cultures at 500 cells per well supplemented with ascorbic acid (50 µg/ml). Cocultures were maintained for 10 to 14 days before fixation for immunofluorescence or transmission electron microscopy. DRGN cultures alone and DRGNs cocultured with NSFCs without transfection served as controls.

Electron microscopy

Cocultures (10 to 15 DIV) were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 at 4°C for 4 hours. After treatment with 1% osmium tetroxide, dehydration in an ethyl alcohol series, and embedment, selected areas were mounted, sectioned, stained with 1% uranyl acetate and lead citrate, and examined with an electron microscope (Spoerri et al., 1990). The nature of the axonal or NSFCs process contacts was examined to determine if the transfected cells would ensheath the axons.

Statistics

Statistical analysis (Graph pad Prism) was carried out using ANOVA (significance level, $p < .05$). Cells (500 to 1,000) were sampled systematically from standardized microscopic fields (total magnification, x 200) of cells stained for each

marker. The mean and standard deviation of triplicate samples repeated a minimum of three times was determined for each of the two NSFC lines. Because there were no detectable differences between the two cell lines, data have been reported without reference to which line was evaluated.

Table 5 Antibodies and specificity

Antibodies	Target	Source
Nestin, human monoclonal, 1:100	Neural stem cells	Chemicon International, Temecula, CA
Peripherin, polyclonal, 1:100	Neural crest cells	Chemicon
β -tubulin III, monoclonal, 1:100	Neurons and progenitors	Chemicon
Neural cell adhesion molecule (NCAM), human monoclonal, 1:100	Neurons and precursors	Chemicon
A2B5, monoclonal, 1:100	Glial and neuronal precursors	Chemicon
2'3'-Cyclic nucleotide-3'-phosphohydrolase (CNP)	Oligodendrocyte precursors	Chemicon
NeuN, monoclonal, 1:100	Neurons	Chemicon
Glial fibrillary acidic protein (GFAP), monoclonal and polyclonal, 1:100	Astrocytes	Chemicon
OX42, monoclonal, 1:100	Microglia	Chemicon
O4, monoclonal, 1:100	Oligodendrocyte precursors	Chemicon
Galactosylceramide (GalC), polyclonal, 1:100	Oligodendrocyte precursors	Chemicon
RIP, monoclonal, 1:1000	Oligodendrocytes	Chemicon
Myelin basic protein (MBP), human polyclonal, 1:200	Oligodendrocytes	Chemicon
Olig2, polyclonal, 1:1000	Transcription factor	Drs. David H. Rowitch and Charles D. Stiles
Nkx2.2, monoclonal, 1:10	Transcription factor	Developmental Studies Hybridoma Bank, (DSHB) Iowa City, IA
Sox10, polyclonal, 1:100	Glial precursors, and oligodendrocytes	Chemicon

RESULTS

NSFC population

The heterogeneous nature of the NSFC population just before transfection was demonstrated by immunolocalization on fixed triton-treated cells. More than 97% ($n = 54$ fields) of cells were positive for β -tubulin III and peripherin (Figs. 7I, 1J); $49.1 \pm 2.9\%$ were positive for nestin (Figs. 7E, 7M) in the absence of triton; $26.1 \pm 1.7\%$ were positive for A2B5 (Fig. 7J); and $40.5 \pm 2.3\%$ were positive for neuronal cell adhesion molecule (NCAM) (Fig. 7K). In contrast, no cells were detected that were reactive for O4, galactosylceramide (GalC), oligodendrocyte specific molecule (RIP), MBP, Glial fibrillary acidic protein (GFAP), neuronal nuclear marker (NeuN), Olig2, Nkx2.2, or Sox10. No differences in phenotypic expression were detected between the two lines selected for these studies.

NSFCs transfected with *Olig2* and *Nkx2.2* exhibit oligodendrocyte-like morphologies

To examine the phenotypic expression of NSFCs *in vitro* after transfection and selection, representative cultures and controls were compared. Three different NSFC morphologies were observed when nontransfected cells were maintained in DFB27 medium: round, bipolar, or multipolar with relatively few processes (0DIV, 9 DIV; Figs. 7A, 7L, respectively). Nontransfected NSFCs or those transfected with lipofectamine died within 1 week after selection with 50 μ g/ml G418. Transfection with single genes or the control vectors resulted in no morphologic changes as indicated for *Olig2*, *Olig2-EGFP*, or *Nkx2.2-EGFP* alone (Figs. 8A-8C). In contrast, the morphology of NSFCs transfected with both *Olig2* and *Nkx2.2* and selection with

G418 for 7 days underwent dramatic changes that resulted in a phenotype characteristic of oligodendrocytes with extensive arborization (Fig. 7D). Quantitative analysis demonstrated not only that NSFCs transfected with both *Olig2* and *Nkx2.2* had increased numbers of processes but also that the processes were longer (Figs. 9A, 9B). After 1 week of selection with G418, several of the cells in the transfected groups were round or had only one process and contained condensed or degraded nuclei. These cells likely reflected a population of nontransfected cells undergoing apoptosis. Because they remained attached to the surface, they were included in the process formation assay. Relatively few NSFCs ($10.4 \pm 0.3\%$, $n = 54$ fields) survived after transfection with vector alone, *Olig2*, *Nkx2.2* or *Olig2* and *Nkx2.2*, and 7-day selection. However, no difference in viability was observed between cells that survived after 2 days of transfection and 7 days of selection and controls without transfection and selection, as determined by a cytotoxicity assay (Fig. 10).

NSFCs transfected with *Olig2* and *Nkx2.2* express oligodendrocyte lineage-restricted markers

Immunohistochemistry of *Olig2*-expressing cells revealed that $48.1 \pm 5.1\%$ of the cells coexpressed nestin (Fig. 8B). On further analysis, no coexpression of O4, GalC, oligodendrocyte specific molecule (RIP), MBP, GFAP, neuronal nuclear marker (NeuN), or OX 42 was noted in the transfected cells (Table 6). Similarly, no detectable lineage-restricted changes were observed in NSFCs transfected with *Nkx2.2*, *Sox10* cDNA, or control vectors alone (Table 6).

However, when the NSFCs were transfected with *Olig2* and *Nkx2.2* simultaneously, morphologic and lineage-restricted changes occurred.

Immunohistochemistry confirmed that the cotransfected cells expressed both *Olig2* and *Nkx2.2* proteins (Fig. 11A). Interestingly, the early oligodendrocyte precursor marker O4 was not expressed in the cotransfected cells. Instead, these cotransfected cells expressed more mature oligodendrocyte markers, including 2'3'-cyclic nucleotide-3'-phosphohydrolase (CNP), GalC, RIP, and MBP (Figs. 11B-11D; Table 6). In addition, the transfected cells coexpressed GalC and CNP (Fig. 11E) as well as RIP and MBP (Fig. 11F). Thus, the presence of the transcription factors *Olig2* and *Nkx2.2* is sufficient to direct differentiation of the human NSFCs toward an oligodendrocytic phenotype. No mature markers for other cell lineages were detected, including GFAP (astrocyte marker), NeuN (neuronal marker), or OX 42 (microglia marker) (Table 6).

The oligodendrocytic phenotype induced by the transcription factors was further confirmed by Western blot analyses with antibodies that recognize the late oligodendrocyte marker MBP. In NSFCs cultured in defined medium (DFB27) or transfected by *Olig2*, *Nkx2.2*, *Sox10*, or control vectors alone, MBP bands were not present (Figs. 12C, 12E). However, in the cells cotransfected with both *Olig2* and *Nkx2.2*, these bands were detected, indicating that only those cells transfected with these two transcription factors produced MBP (Figs. 11D, 11F, 12C, 12E).

Time course analysis of antigen expression

To examine the changed pattern of antigen expression by NSFCs transfected with *Olig2-Nkx2.2*, NSFCs before transfection (0 DIV) and after 2 days transfection and 7 days selection (3, 6, and 9 DIV) were fixed for immunohistochemistry. Although some cells within the initial population of NSFCs in DFB27M were A2B5-

positive, this antigen was not examined further because it may be unreliable for specific glial restriction since it has been reported to cross-react with neurons (Spoerri et al., 1988). Therefore, the expression of one early, O4, an early oligodendrocyte marker, and several relatively mature oligodendrocyte markers, including CNP, GalC, RIP, and MBP, was examined. Most NSFCs were round, and a few were bipolar or multipolar (Figs. 7A, 7L); 49% of the NSFCs expressed nestin, which is expressed in neural stem cells, progenitors, or proliferating oligodendrocyte progenitors (Gallo et al., 1995); and > 97% of the NSFCs were peripherin-positive (Fig. 7E) before transfection (0 DIV). After transfection with *Olig2-Nkx2.2*, NSFCs formed elaborate compound processes (Figs. 7B-7D) and gradually lost their expression of nestin (Fig. 7M); furthermore, a few cells began to express CNP and GalC on 3 DIV. By 6 DIV, most cells were positive for these two antigens (Figs. 7F, 7M). It was not until 9 DIV that > 85% of the cells expressed the more mature oligodendrocyte markers RIP and MBP (Figs. 7G, 7M). At no time point was expression of NeuN, GFAP (Figs. 7H, 7M), O4, and OX 42 (Fig. 7M) observed (0 DIV to 9 DIV).

Sox10* can mimic the effects of *Olig2* in inducing oligodendrocyte phenotype in collaboration with *Nkx2.2

Recent studies have suggested that *Sox10* is the downstream target gene of *Olig2* (Poncet et al., 1996; Xu et al., 2000) and may mediate the function of *Olig2* in regulating oligodendrocyte specification and differentiation. Thus, the expression of *Sox10* in NSFCs during the induction by *Olig2* and *Nkx2.2* was examined. When the NSFCs were transfected with *Olig2* alone, $9.4 \pm 0.8\%$ of the transfected cells expressed *Sox10* (Table 6). In contrast, $25.5 \pm 1.8\%$ of the NSFCs transfected with

both *Olig2* and *Nkx2.2* expressed Sox10 (Table 6). To test the role of *Sox10* in oligodendrocyte induction, NSFCs were transfected with *Sox10-EGFP* alone or both *Sox10* and *Olig2*; no oligodendrocyte morphology was detected (Figs. 13A, 13B; Table 6). However, when *Sox 10* and *Nkx2.2* were introduced into the cells simultaneously, the cells assumed the characteristic phenotype of the oligodendrocytes (Fig. 13C; Table 6).

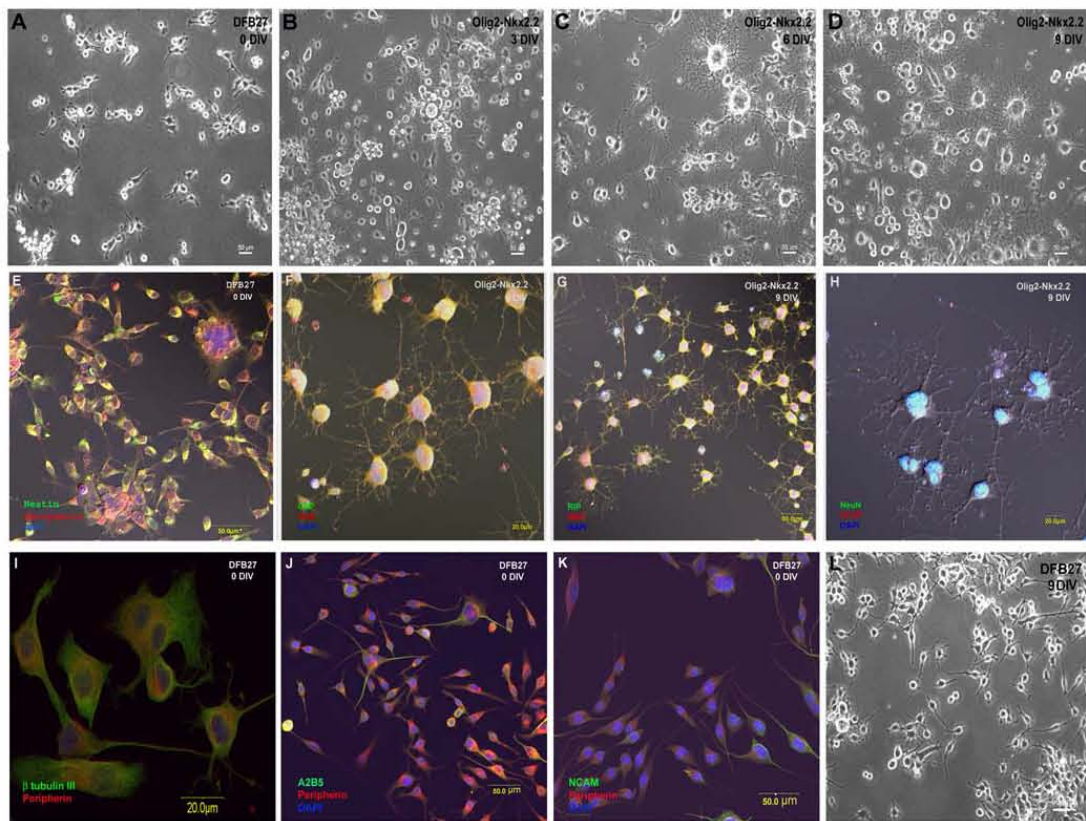
The induction of Sox10 protein in the NSFCs transfected with various expression vectors was further verified by Western blot (Figs. 12D, 12E). When the cells were transfected with only *Nkx2.2* or the control vectors or grown in DFB27M, Sox10 protein expression was not detected. However, Sox10 protein was detected in cells transfected with either *Olig2* alone or with *Olig2* and *Nkx2.2* simultaneously and at a much higher level in cells that were transfected with *Sox10* alone or in combination with *Nkx2.2* (Fig. 12D; Table 6). In addition, several bands of smaller sizes were detected, which were probably degradation products of the Sox10 protein (Fig. 12D).

Cocultured of NSFCs transfected with *Olig2-Nkx2.2* or *Sox10-Nkx2.2* with purified rat or GFP mouse DRG neurons

No direct axonal-NSFC association was observed when non-transfected NSFCs were maintained on top of an established DRG neuronal layer (controls) for 10 to 14 days. In contrast, NSFCs transfected with *Olig2-Nkx2.2* or *Sox10-Nkx2.2*, cocultured with DRGNs for 10 to 14 days, formed multiple processes that often were observed in direct contact with the DRG neurites. As demonstrated with confocal microscopy, NSFC processes were observed, wrapping around individual regions of the DRG

neurites (Figs. 14A, 14B). The transfected cells were MBP-positive. Pilot ultrastructural analysis demonstrated the early stages of axonal ensheathment by processes from the transfected NSFCs (Figs. 15A-15C). Frequent regions of subplasmalemmal densities were observed at the contact sites between axons and the NSFC processes, perhaps reflecting the initial development of mesaxons.

Figure 7. Time course of phenotype and lineage changes. Adult human olfactory progenitors (NSFCs, passage 12) before transfection (0 DIV) or without transfection for 9 DIV (L) did not exhibit an oligodendrocyte-like phenotype. (A): Immunolocalization demonstrated > 97% β -tubulin III⁺ (green) and peripherin⁺ (red) (I); 26% A2B5⁺ (green, J); 41% NCAM⁺ (green, K); and 49% nestin⁺ (green, E) of the NSFC population (E). During transfection with *Olig2-Nkx2.2* and selection, NSFCs began to form networks of extensive processes (B: 3 DIV; C: 6 DIV; D: 9 DIV). Furthermore, approximately 80% of cells gained CNP (green) and GalC (red) expression on 6 DIV (F, M), and most lost nestin expression (M); on 9 DIV, most cells (> 85%) gained RIP (green) and MBP (red) expression (G, M). There was no expression of NeuN (green), GFAP (red) (H, M), and OX 42 (M) 4', 6-diamidino-2-phenylindole dihydrochloride (blue stain for DNA). (A-D, L): Phase-contrast optics; (E-K): Confocal microscopy, differential interference contrast (DIC). Data are expressed as mean \pm standard deviation, $n = 54$ fields. Each experiment included triplicate samples, repeated a minimum of three times. Abbreviations: CNP, 2'3'-Cyclic nucleotide-3'-phosphohydrolase; DIV, days in vitro; GalC, galactosylceramide; GFAP, Glial fibrillary acidic protein; MBP, myelin basic protein; NSFC, neurosphere-forming cell.



Time Course Analysis of Antigen Expression

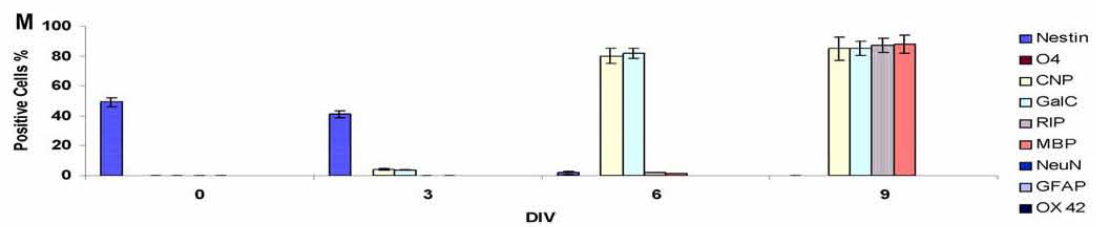


Figure 8. NSFCs (passage 10 through 20) transfected with either *Olig2* or *Nkx2.2* did not exhibit oligodendrocyte-like morphology. (A): Immunolocalization demonstrated *Olig2* expression (red) in 5% to 10% of the NSFCs after 2 days of transfection with *Olig2*. (B): After 2 days of transfection with *Olig2-EGFP* and 7 days of selection, the transfected NSFCs appeared similar to nontransfected controls. Immunoreactivity to nestin (red) was also similar to control populations. (C): Transfection with *Nkx2.2-EGFP* for 2 days followed by 7 days of selection did not alter NSFC morphology of cells reactive for *Nkx2.2* (green). (Confocal microscopy, A and B, differential interference contrast (DIC). Abbreviations: DIV, days in vitro; NSFC, neurosphere-forming cell.

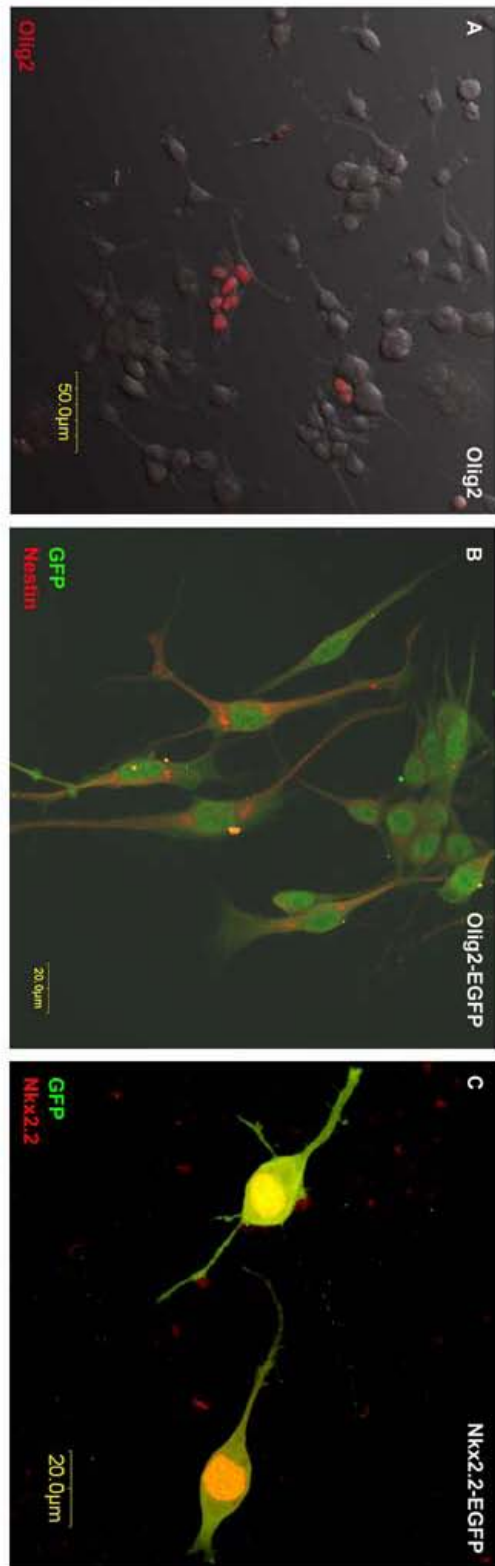


Figure 9. Time-dependant changes in process number (A) and average length (B) between 1 and 9 days of transfection and selection. Transfection with both *Olig2* and *Nkx2.2* (*Olig2-Nkx2.2*) or *Sox10* and *Nkx2.2* (*Sox10-Nkx2.2*) increased the number and length of processes per cell ($p < 0.01$). The decrease in number of processes per cell in the DFB27 group reflects the high proliferative activity of NSFCs on the coverslips at 7 DIV. Values are mean \pm standard deviation. $**p < 0.01$ (t -test). pIRES as the control vector, NSFCs without transfection (DFB27), transfected with *Olig2* with *EGFP* (*Olig2-EGFP*), *Nkx2.2* with *EGFP* (*Nkx2.2-EGFP*), and *Sox10* with *EGFP* (*Sox10-EGFP*) alone. Abbreviations: DIV, days in vitro; NSFC, neurosphere-forming cell.

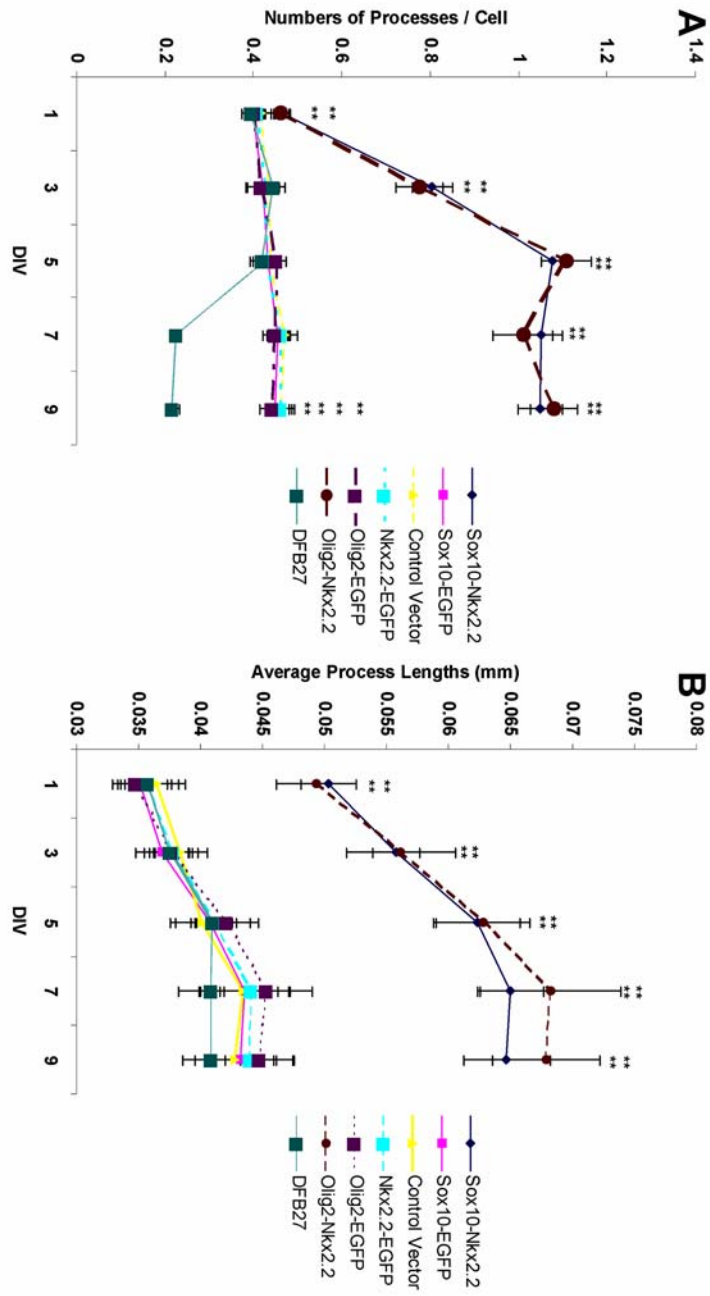


Figure 10. MTT assay. NSFCs (passage 10 through 20) were cultured in DFB27M without transfection and selection (DFB27) or transfected with control vector, *Olig2-EGFP*, *Nkx2.2-EGFP*, *Sox10-EGFP*, *Olig2-Nkx2.2*, and *Sox10-Nkx2.2* for 2 days, selected for 7 days, and assayed for viability using the MTT assay. No differences were observed between the groups. Data are expressed as mean \pm standard deviation ($n = 12$). Each experiment includes triplicate samples. All experiments were repeated a minimum of three times. Abbreviations: DIV, days in vitro; NSFC, neurosphere-forming cell.

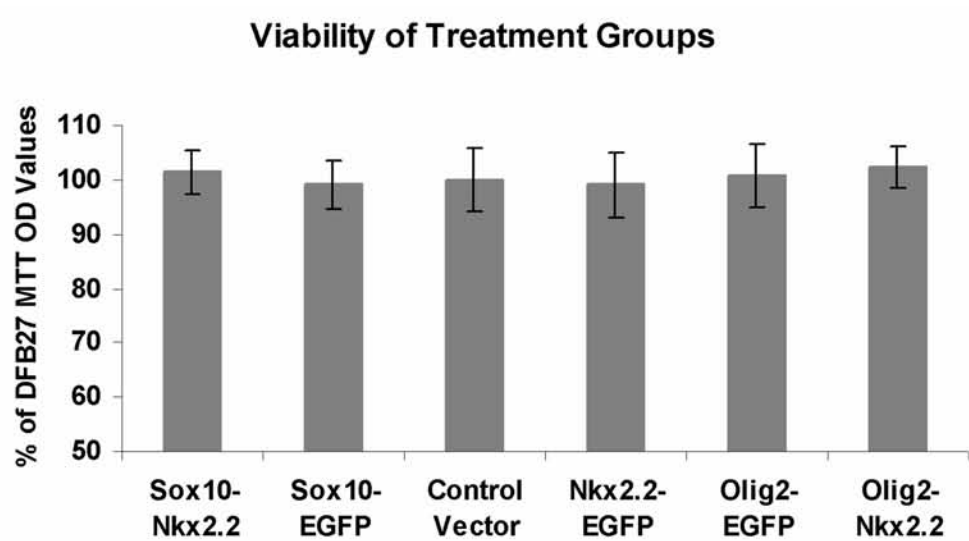


Table 6. Analysis of the presence of oligodendrocyte markers in NSFCs after transfection

<i>NSFCs</i>	O4 + %	CNP + %	GalC + %	RIP + %	MBP + %	Sox10 + %	GFAP + %	Neu N+ %	OX-42 +
Control	0	0	0	0	0	0	0	0	0
DFB27+ Lipo	0	0	0	0	0	0	0	0	0
PIRES2-EGFP	0	0	0	0	0	0	0	0	0
pIRES	0	0	0	0	0	0	0	0	0
<i>Olig2</i> -EGFP	0	0	0	0	0	9.4 ±0.8	0	0	0
<i>Nkx2.2</i> -EGFP	0	0	0	0	0	0	0	0	0
<i>Sox10</i> -EGFP	0	0	0	0	0	>95%	0	0	0
<i>Olig2</i> - <i>Nkx2.2</i>	0	84.9 ±7.8	85.2 ±4.9	86.9 ±4.8	87.9 ±5.9	25.5 ±1.7	0	0	0
<i>Olig2</i> + <i>Sox10</i> -EGFP	0	0	0	0	0	>95%	0	0	0
<i>Sox10</i> - <i>Nkx2.2</i>	0	84.2 ±8.2	86.1 ±9.0	86.8 ±7.6	89.2 ±7.6	>95%	0	0	0

NSFCs (passage 10 through 20, cultured in DFB27M) transfected with lipofectamine only (DFB27 + Lipo), pIRES2-EGFP vector, pIRES vector, pIRES2-EGFP with *Olig2* vector (*Olig2*-EGFP), pIRES2-EGFP with *Nkx2.2* vector (*Nkx2.2*-EGFP), pIRES2-EGFP with *Sox10* vector (*Sox10*-EGFP), pIRES with *Olig2* + *Nkx2.2* vector (*Olig2*-*Nkx2.2*), and pIRES with *Sox10* + *Nkx2.2* vector (*Sox10*-*Nkx2.2*) for 2 days after 7 days of selection and then fixed for immunocytochemistry analysis. NSFCs cultured 9 days in DFB27 without transfection served as the control. Values are means expressed as percentages ± standard deviation (n = 54 fields). Each experiment included triplicate samples and was repeated a minimum of three times.

Abbreviations: CNP, 2'3'-cyclic nucleotide-3'-phosphohydrolase; GalC, galactosylceramide; GFAP, Glial fibrillary acidic protein; NSFC, neurosphere-forming cell.

Figure 11. Cotransfection of *Olig2* and *Nkx2.2* resulted in lineage change. After 2 days of simultaneous transfection with *Olig2* and *Nkx2.2* and 7 days of selection, NSFCs (passage 10 through 20) exhibited a characteristic oligodendrocytic phenotypic expression. These representative micrographs illustrate immunolocalization with probes for individual transcription factors (B, C, D) or both transcription factors (A) as well as for several oligodendrocyte specific lineage-restricted antigens (E, F). The specific primary antibodies have been noted on the respective micrographs. They were demonstrated with secondary antibodies labeled with CY2 (green) or Texas red (red). The confocal images included a DIC channel to demonstrate the extent of phenotypic expression. (A): NSFCs immunostained for both transcription factors, *Nkx 2.2* (green) and *Olig2* (red). (B): Approximately 85% of the NSFCs transfected with both transcription factors were immunoreactive for oligodendrocytic-specific antigens GalC (red), RIP (green) (C), and human MBP (red) (D). The simultaneous presence of two independent oligodendrocyte-specific antigens, CNP (green) and GalC (red) (E), as well as oligodendrocyte specific molecule (RIP) (green) and MBP (red) (F), was demonstrated in cells transfected with both transcription factors. Abbreviations: CNP, 2'3'-Cyclic nucleotide-3'-phosphohydrolase; DIV, days in vitro; GalC, galactosylceramide; MBP, myelin basic protein; NSFC, neurosphere-forming cell.

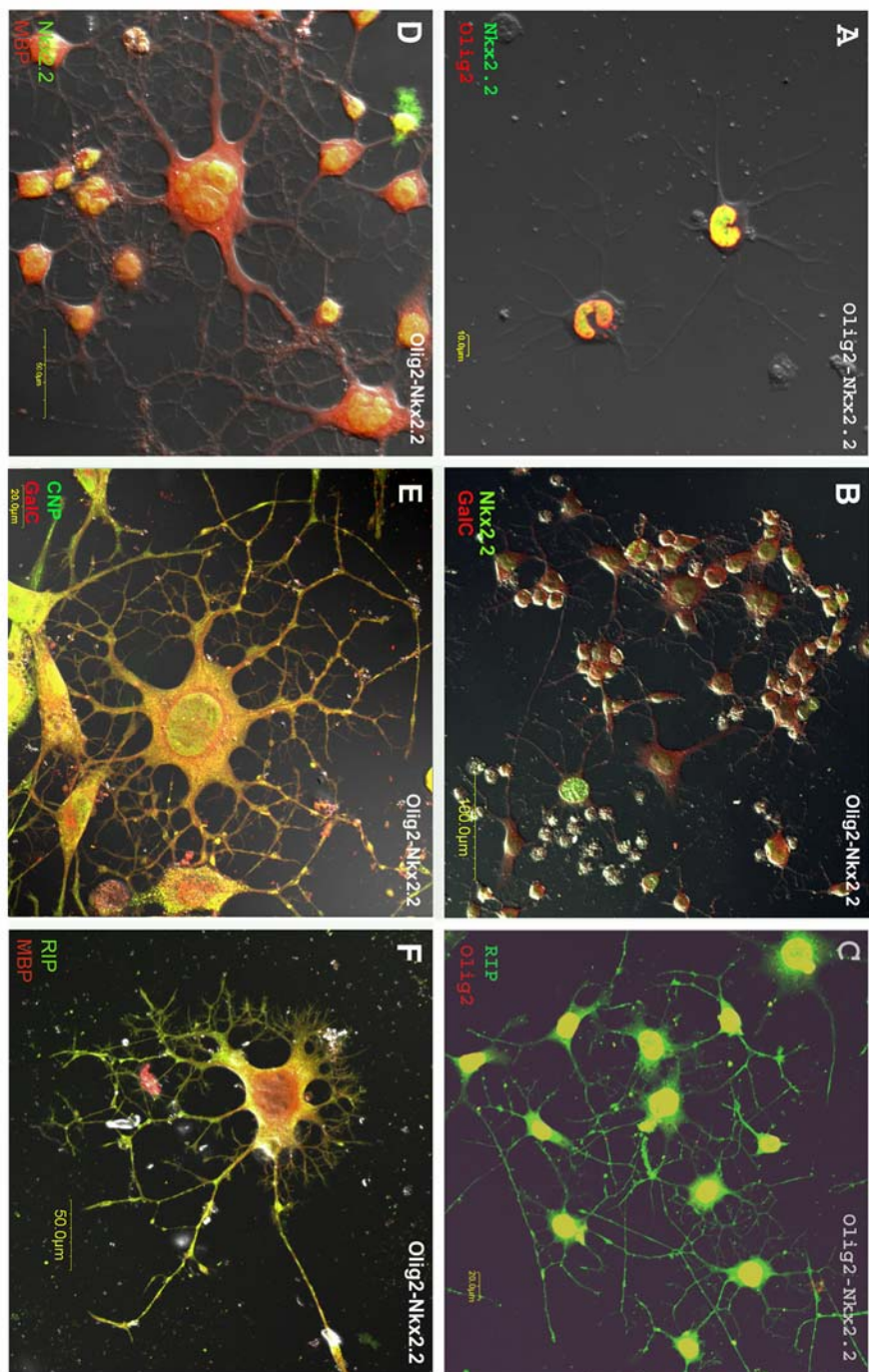


Figure 12. Western blot assay. After 2 days of transfection and 7 days of selection, NSFCs (passage 10 through 20) were lysed in buffer; protein samples were separated on SDS-PAGE gels, and the expression of Olig2, Nkx2.2, human MBP, and *Sox10* was detected. Human fibroblasts and NSFCs cultured in defined medium (DFB27) served as the controls. Actin was used as a control for variation in cell density. (A): There was endogenous Olig2 expression in NSFCs, but not human fibroblasts. (B): No endogenous Nkx2.2 expression was detected in either NSFCs or human fibroblasts. (C): NSFCs transfected with *Olig2* and *Nkx2.2* or *Sox10* and *Nkx2.2* expressed human MBP. (D): NSFCs transfected with *Olig2* alone, *Olig2* with *Nkx2.2*, *Sox10* alone, or *Sox10* with *Nkx2.2* expressed *Sox10*. (E): Quantification of protein bands expressed as mean \pm standard deviation. The density of the actin band was used as standard to adjust tracing quantification. The pIRES2 expression vector served as the control vector. Abbreviations: MBP, myelin basic protein; NSFC, neurosphere-forming cell.

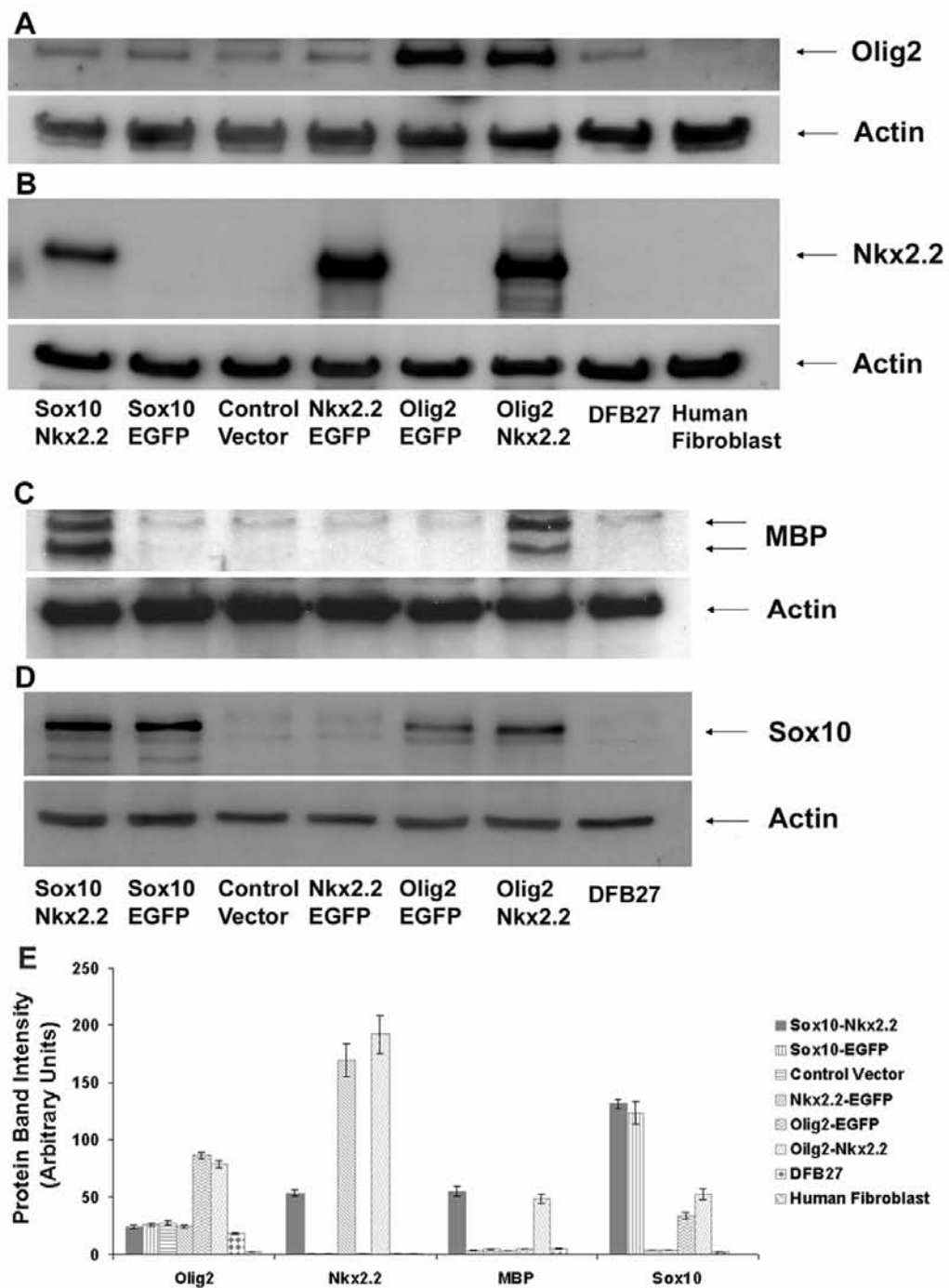


Figure 13. Transfection with *Sox10* alone or with *Nkx2.2* simultaneously. The NSFCs (passage 10 through 20) after 2 days of transfection with *Sox10* with *EGFP* alone (A), *Olig2* and *Sox10* with *EGFP* (B), or *Sox10* with *Nkx2.2* (C) and 7 days of selection expressed (A) *Sox10* (red), (B) *Olig2* (red), and (A, B) GFP (green) or (C) *Nkx2.2* (green). Furthermore, NSFCs transfected simultaneously with *Sox10* and *Nkx2.2* (C) were immunoreactive for MBP (red) and phenotypically characterized by extensive arborization. Abbreviations: MBP, myelin basic protein; NSFC, neurosphere-forming cell.

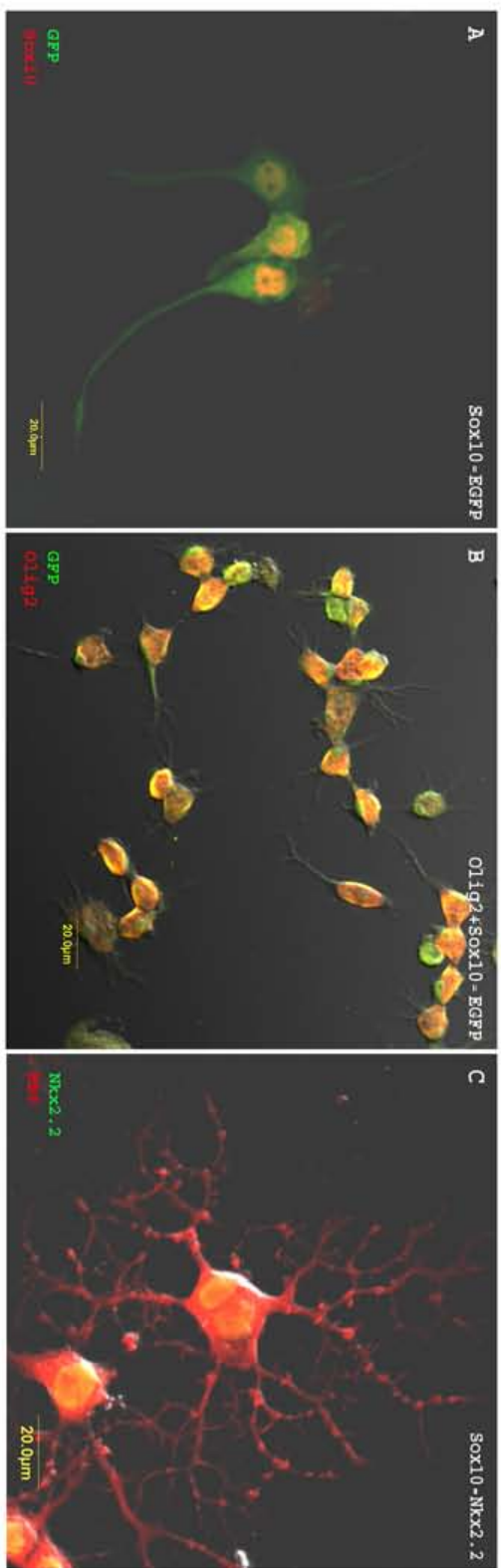


Figure 14. Coculture studies. After 2 days of transfection (*Olig2-Nkx2.2*, *Sox10-Nkx2.2*) and 7 days of selection, NSFCs (passage 16) were maintained in DFB27M without G418 for 1 day and then seeded onto purified GFP mouse DRGNs for 11 days. The NSFCs were MBP (red) positive; their processes were frequently found surrounding individual axonal segments of DRGNs (arrow). (A): Cells transfected with *Olig2-Nkx2.2*; (B): Cells transfected with *Sox10-Nkx2.2*. Abbreviations: DRGN, dorsal root ganglia neuron; GFP, green fluorescent protein; MBP, myelin basic protein; NSFC, neurosphere-forming cell.

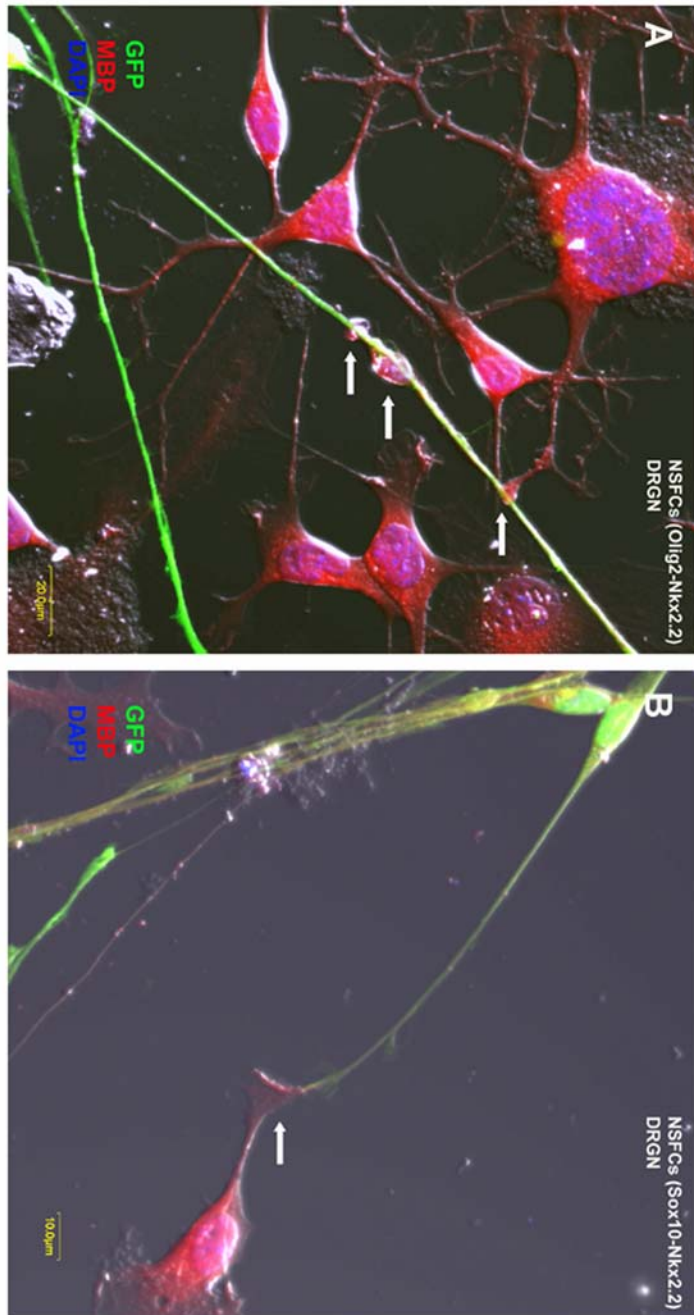
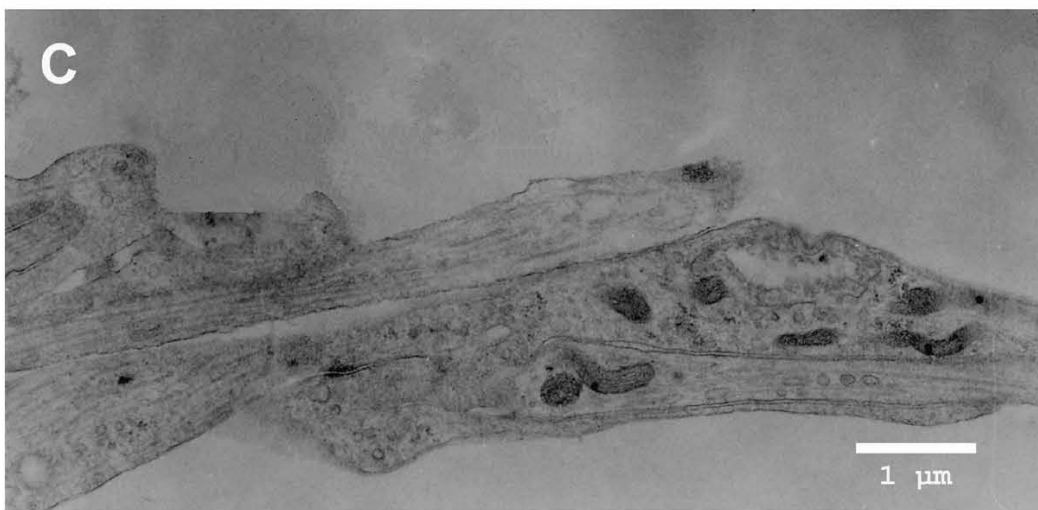
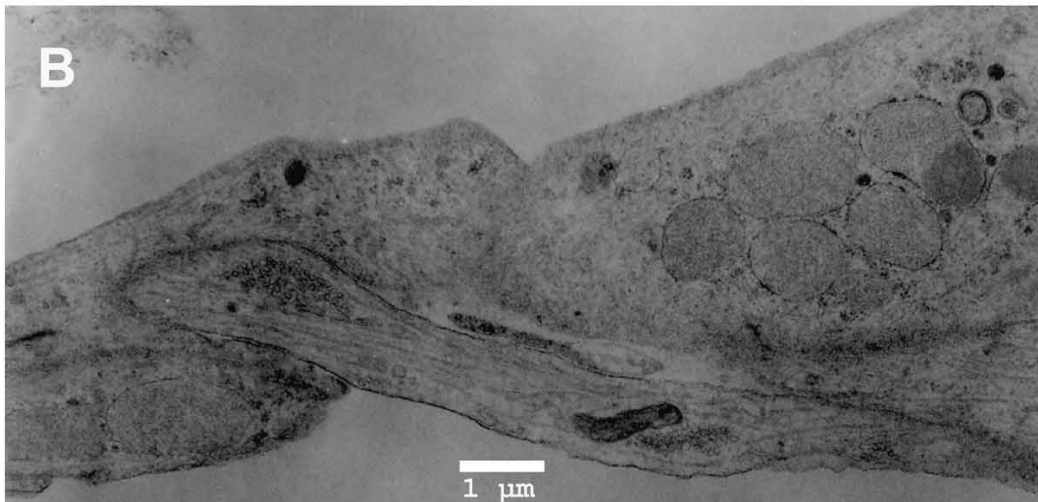
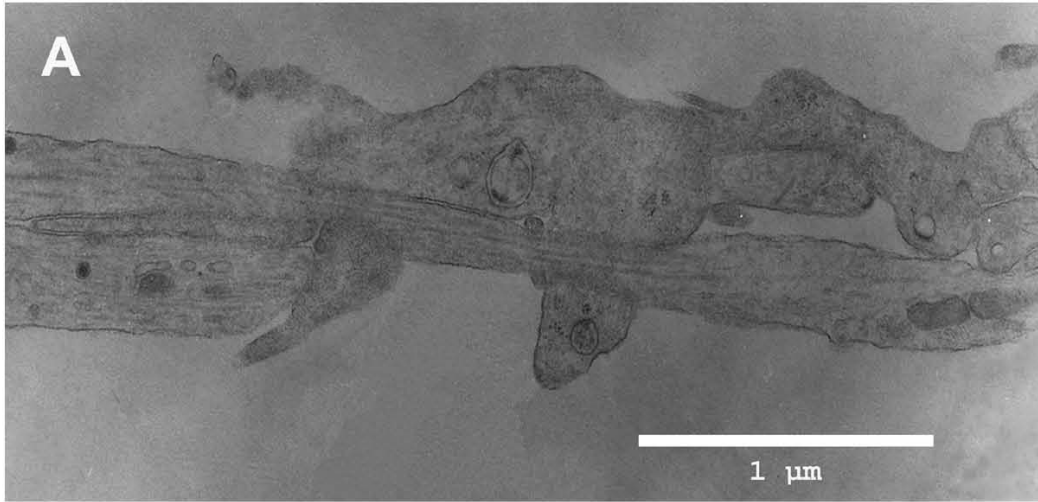


Figure 15. Ultrastructural observation of transfected NSFCs cocultured with rat DRGNs. After transfection (*Olig2-Nkx2.2*) and selection, GFP-labeled NSFCs (passage 15) were maintained in DFB27M without G418 for 1 day and then seeded onto purified rat DRGNs for 11 days. (A): Two processes of transfected NSFCs wrapped around the axon of the DRGN. (B): One larger process of transfected NSFCs ensheathed the axon of the DRGN. (C): The processes of a transfected NSFC ensheathed a middle axonal segment. Abbreviations: DRGN, dorsal root ganglia neuron; NSFC, neurosphere-forming cell.



DISCUSSION

Neural stem cells can give rise to a variety of neuronal and glial phenotypes in culture and therefore have the potential to provide a cell population for replacement-based therapeutic treatments for various neurological diseases or injuries (Chandran et al., 2004; Gage, 2000; McKay, 1997; Svendsen et al., 1996). The human NSFCs from adult olfactory epithelium cultured in MEM with 10% FBS were shown to remain relatively undifferentiated despite exposure to a variety of media and trophic factors (Roisen et al., 2001; Zhang et al., 2004). In fact, this progenitor population seems to have an immature neuronal default. This suggests that the NSFCs obtained from adult human olfactory epithelium may be different from embryonic or other species of neural stem or progenitor cells (Chandran et al., 2004). In the present study, during transfection-mediated oligodendrocyte differentiation, NSFCs gradually lost their progenitor characteristics, such as reactivity to nestin as previously reported for other cells (Gallo et al., 1995), and gained the properties of mature oligodendrocytes, bypassing the earliest stages of oligodendrocyte differentiation. A few CNP⁺ or GalC⁺ but MBP⁻ oligodendrocytes were observed that exhibited two to three processes without the web-like morphology, suggesting that they were premature or immature oligodendrocytes. However, we did not detect any O4, a marker for a specific preoligodendrocyte developmental stage. One possible explanation is that overexpression of *Olig2* or *Nkx2.2* could modify cell surface properties or the overexpression of the factors could result in a rapid maturation and our timing for evaluation of the cells' differentiation missed a very brief period of O4 expression.

Another explanation is that *NKx2.2* may directly activate MBP gene expression in collaboration with *Olig2* (Qi et al., 2001) without activating *O4* expression.

Neural stem cells have been shown to differentiate into neurons or glia after exposure to growth factors or mitogen withdrawal (Gage, 2000; McKay, 1997; Svendsen et al., 1996). Recent identification of many key lineage-specific molecules has provided important insights into directing progenitors to differentiate into the desired specific cell types. Molecular and genetic studies have shown that many transcription factors, when expressed in combination, are capable of driving lineage-specific differentiation during embryonic development (Marquardt and Pfaff, 2001). For instance, in the developing chick spinal cord, coelectroporation of *Olig2* and *Nkx2.2* promoted oligodendrocyte differentiation and maturation (Zhou et al., 2001), whereas expression of either *Olig2* or *Nkx2.2* alone was not sufficient to cause such differentiation. In this study, *Olig2* and *Nkx2.2* have been used to drive adult human progenitors to differentiate along an oligodendrocyte lineage. Consistent with the previous observations in embryonic neural stem cells, neither *Olig2* nor *Nkx2.2* could induce NSFCs to differentiate into oligodendrocytes. However, NSFCs transfected simultaneously with *Olig2* and *Nkx2.2* exhibited oligodendrocyte morphology and antigen expression, resembling *in vivo* patterns. Western blot analysis provided parallel independent assessment to complement the immunolocalization. The two different approaches demonstrated that *Olig2* and *Nkx2.2* or *Nkx2.2* and *Sox10* but not *Olig2* or *Nkx2.2* possessed the capability of driving oligodendrocyte lineage-specific differentiation. Interestingly, the expression of *Olig2* gene in avian olfactory epithelium from E11.5 onward has been reported (Zhou et al., 2001). However, no

expression of *Olig2* or *Nkx2.2* was detected in the adult human olfactory epithelial-derived NSFCs by immunocytochemistry. Western blot analysis further demonstrated the absence of endogenous *Nkx2.2* expression. In contrast, a low level of endogenous *Olig2* expression was detected in the NSFCs. This suggests that the low level of endogenous *Olig2* expression was not sufficient to cooperate with *Nkx2.2* to induce NSFC differentiation into oligodendrocytes.

In the present study, the mechanism of induction of oligodendrocytes by *Olig2* and *Nkx2.2* was investigated. Previous studies indicated that overexpression of *Olig2* alone *in ovo* induced the expression of *Sox10* but not other oligodendrocyte-specific transcription factors, such as *Nkx2.2* (Zhou et al., 2000; 2001; Sun et al., 2001). The Sox proteins represent a family of high-mobility group-containing transcription factors. So far, at least two Sox proteins are known to be involved in the development of myelin-forming oligodendrocytes. Sox9 regulates oligodendrocyte specification, whereas Sox10 is required for terminal differentiation (Stolt et al., 2002; Kuhlbrodt et al., 1998; Britsch et al., 2001; Stolt et al., 2003). These findings suggest that *Sox10* may function downstream of *Olig2* and mediate the function of *Olig2* in collaboration with *Nkx2.2* to control oligodendrocyte differentiation and maturation. In agreement with this observation, NSFCs transfected with *Olig2* alone expressed Sox10, although only a small percentage of transfected NSFCs were able to respond to *Olig2* by the induction of *Sox10*. However, NSFCs transfected with *Olig2* and *Nkx2.2* together induced more Sox10, consistent with a previous report that an interaction between *Olig2* and *Nkx2.2* could promote *Sox10* expression in chick neural tube-generated cells (Sun et al., 2001). Consistent with the concept that *Sox10* functions downstream

of *Olig2*, overexpression of *Sox10* and *Nkx2.2* in NSFCs achieved equivalent effects compared with cells with *Olig2* and *Nkx2.2*. Expression of *Sox10* in conjunction with *Nkx2.2* resulted in myelin gene expression, which is important for terminal oligodendrocyte differentiation (Stolt et al., 2002; Peirano et al., 2000; Slutsky et al., 2003). NSFCs expressing *Olig2* or *Sox10* alone did not express the myelin gene or differentiate into oligodendrocytes, possibly because *Sox10* itself is a weak transcriptional activator and needs to exert its function in concert with other transcription factors, such as *Nkx2.2* (Kuhlbrodt et al., 1998; Kamachi et al., 2000).

In vivo evidence revealed that oligodendrocyte progenitors express both Sox9 and Sox10 and can cope with loss of either protein. In *Sox10* knockout mice, a few residual MBP-positive oligodendrocytes are still found in the spinal cord at birth (Stolt et al., 2002; 2003). This raises the possibility that other transcription factors might be involved in the regulation of myelin gene expression as well. Interestingly, in our study, although only 25.5% of NSFCs transfected with *Olig2* and *Nkx2.2* induced *Sox10* expression, more than 84% CNP-, GalC-, RIP-, or MBP-positive cells were found among the transfected cells. This result supports the hypothesis that other unidentified molecules might exist to regulate expression of myelin proteins cooperating with *Nkx2.2* *in vitro*. Those unidentified factors could be the downstream proteins induced by *Olig2* and possess a similar function as *Sox10* in driving NSFCs towards the more mature differentiated oligodendrocyte phenotypes. Together, our results indicated that *Sox10* in adult progenitors could mimic the effects of *Olig2* collaboration with *Nkx2.2* to control myelin gene expression and oligodendrocyte differentiation.

Previously, olfactory ensheathing cells (OECs) (Devon and Doucette, 1995), oligodendrocytes from mouse embryonic stem cells (Liu et al., 2000), and oligodendrocyte precursor cells from 10-day-old rats (Chan et al., 2004) have been reported to form myelin when cocultured with DRGNs. Others report that OECs fail to myelinate axons of DRGNs *in vitro* (Plant et al., 2002) but could form myelin when transplanted to the demyelinated spinal cords of rats (Barnett et al., 2000). In the present study, although NSFCs transfected with *Olig2-Nkx2.2* or *Sox10-Nkx2.2* gained the expression of more mature oligodendrocyte markers and in coculture were competent to initiate axonal ensheathment, they did not form myelin. This may reflect that myelination is a complicated process that requires a specific *in vitro* environment that was not provided by our culture conditions. This possibility is consistent with other reports (Chandran et al., 2004). Future studies will examine the ability of these transfected cells to remyelinate demyelinated regions of rat spinal cord. Furthermore, substantial evidence exists demonstrating differences between species, especially when comparisons between human and rodent cells are undertaken (Chandran et al., 2004).

In summary, these studies reveal, first, that the transcription factors (*Olig2* and *Nkx2.2*) that control oligodendrocytic development in embryonic chick and rodent CNS are able to direct adult human olfactory-derived progenitors towards oligodendrocyte lineage. Second, in this model, *Olig2* and *Nkx2.2* functioned cooperatively to produce oligodendrocyte differentiation. Neither *Olig2* nor *Nkx2.2* alone showed phenotypic changes. Third, NSFCs transfected with *Olig2* or *Nkx2.2* or *Sox10* and *Nkx2.2* expressed oligodendrocyte-specific antigens, including GalC, CNP,

RIP, and human MBP. Fourth, NSFCs transfected with *Olig2* and *Nkx2.2* or *Sox10* and *Nkx2.2* and cocultured with DRGNs gained the capacity to form axonal ensheathments. Furthermore, it was particularly interesting to note that molecular mechanisms that function in early avian and rodent embryonic development, as expected, can be used to drive progenitors derived from individuals 22 or 96 years of age.

The use of transcription factors to modulate adult human olfactory epithelial-derived progenitors to differentiate into specific neuronal or glial cell types may expand the therapeutic potential of these progenitors. This is especially important, because the readily accessible location of adult olfactory neuroepithelium, which does not require highly invasive surgery for its biopsy (Winstead et al., 2005), could provide an autologous progenitor source for cell replacement transplantation strategies. Furthermore, the use of these progenitors would eliminate ethical concerns related to the use of embryonic stem cells, avoid donor availability issues, and ensure complete histocompatibility, thereby negating the need for immunosuppression.

ACKNOWLEDGMENTS

Xiaodong Zhang and Jun Cai contributed equally to this work. The authors thank George Harding for his assistance with the confocal microscopy and Cathie Caple for technical assistance with the electron microscopy. This work was supported by NIH (1920RR15576 to F.J.R.), Kentucky Spinal Cord Head Injury Research Trust (to M.Q.), and National Multiple Sclerosis Society (FA1400-A-1 to J.C.).

CHAPTER IV

INDUCTION OF NEURONAL DIFFERENTIATION OF ADULT HUMAN

OLFACTORY NEUROEPITHELIAL-DERIVED PROGENITORS

THROUGH MAPK AND PI3-K SIGNALING PATHWAYS

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Submitted: J. Neurochem.

Abbreviation list: ACh: acetylcholine, bFGF: basic fibroblast growth factor, BrdU: 5-bromo-2'-deoxyuridine, BSA: bovine serum albumin, CB: cytoskeletal buffer, ChAT: choline acetyltransferase, CREB: cAMP-responsive element binding protein, DA: dopamine, DAPI: 4', 6-diamidino-2-phenylindole dihydrochloride, DFBNM: DMEM/F12 supplemented with 1% B27 and 0.5% N2, DIV: days in vitro, Erk: extracellular signal-regulated kinase, FN: forskolin, GFAP: glial fibrillary acidic protein, Isl1/2: Islet1/2, MAPK: mitogen-activated protein kinase, MBP: myelin basic protein, MEK: MAPK kinase, NF: Neurofilaments, NSFCs: neurosphere forming cells, P: phosphorylated, PK: protein kinase, RA: retinoic acid, RXRs: retinoid X receptors, Shh: sonic hedgehog, T: total, TBS: Tris-Buffered Saline, TH: tyrosine hydroxylase, VAChT: vesicular acetylcholine transporter, WB: Western blot.

INTRODUCTION

The olfactory neuroepithelium is unique compared to other regions of the nervous system; it has the capacity to replace damaged neurons and glia throughout life (Moulton, 1974; Graziadei et al., 1979; Calof and Chikaraishi, 1989; Sosnowski et al., 1995; Zhang et al., 2000). Adult human olfactory neuroepithelium can provide a unique source of adult neural progenitors, which can be obtained from an individual without invasive surgery (Winstead et al., 2005). Approximately 75 heterogeneous cell lines of neurosphere forming cells (NSFCs) have been established in the lab from primary cultures of adult olfactory neuroepithelium isolated from cadavers (Roisen et al., 2001) or patients (Winstead et al., 2005). Analysis of the NSFCs revealed that these cells have the potential to differentiate along neuronal or glial lineage restriction

depending on environmental signals *in vitro* (Roisen et al., 2001; Zhang et al., 2005). The long term goal of this research is to use these cells as a potential source for autologous cell replacement strategies for the treatment of neurodegenerative diseases (Duncan et al., 1997; Scherer, 1997), or for analysis of gene function and diagnostic testing.

The mechanisms underlying neuronal specification and differentiation from embryonic neural stem cells or progenitors are under extensive investigation. Neuronal differentiation depends on inductive signals such as neurotrophic factors, RA, FN, and Shh. The molecular mechanisms through which RA, FN, and Shh mediate cellular differentiation and growth suppression in neural cells are in early stages of study.

A number of neurotrophic factors have been studied including glial derived neurotrophic factor (GDNF) and brain derived neurotrophic factor (BDNF). GDNF has been shown to promote survival and differentiation of dopaminergic neurons derived from human embryonic stem cells (Buytaert-Hoefen et al., 2004) and mouse enteric neural crest cells (Fu et al., 2004). BDNF increased neuronal differentiation of mouse embryonic neural precursors (Ahmed et al., 1995). However, these agents have no effect on neuronal differentiation of NSFCs (Zhang et al., 2004), suggesting that other factors may regulate their differentiation. One candidate factor is retinoic acid (RA) which has an important role in the fate specification and differentiation of specific neuron subtypes in the developing CNS (Novitch et al., 2003; Diez del Corral et al., 2003); neurite growth of adult mouse DRG (Corcoran and Maden, 1999); and synaptic plasticity in the adult mouse hippocampus (Misner et al., 2001). Neuronal

development of chick embryonic neural plate explants *in vitro* and *in vivo* were inhibited by blocking RA signaling (Diez del Corral et al., 2003). Recent reports demonstrate that RA has a number of pathways through which it can effect cellular differentiation. RA effects transcription through its specific nuclear receptors (RARs) and / or retinoid X receptors (RXRs) (Canon et al., 2004). There are also reports that RA signaling involves PKC (Gruber et al., 1995), PI3-K/PKB (Lopez-Carballo et al., 2002), mitogen-activated protein kinase (MAPK) (Okamoto et al., 2000), and PKA pathways (Canon et al., 2004), and the cAMP-responsive element binding protein (CREB) (Lonze and Ginty, 2002).

Another important developmental factor that regulates neuronal specification and differentiation is the sonic hedgehog (Shh) molecule. Shh signaling is critical for the generation of neuronal cell types (such as motoneurons and interneurons) in the ventral region of the embryonic chicken CNS (Roelink et al., 1995; Ericson et al., 1997). During early neural development, Shh functions as a morphogen to induce distinct groups of ventral interneurons and motoneurons in a concentration-dependent manner (Briscoe et al., 2000). In addition, Shh can induce motoneurons and dopaminergic neurons from mouse embryonic stem cells (Wichterle et al., 2002; Perrier et al., 2004). Shh has also been reported to promote proliferation of embryonic mouse multipotent enteric neural crest cells (Fu et al., 2004) and the survival of CNS neurons (Rowitch et al., 1999). The effects of Shh on cell fate specification and the proliferation of neuronal precursors appear to be mediated by inhibition of cAMP-dependent protein kinase A (PKA) pathway (Wechsler-Reya and Scott, 1999).

Therefore, the PKA pathway appears to play an important role in mediating RA-and Shh-induced neuronal specification and differentiation. Consistent with this concept, FN, an adenyl cyclase activator that increases intercellular cAMP, can stimulate axonal elongation (Roisen et al., 1972a; Roisen et al., 1972b); enhance neurofilament expression in embryonic rat immortalized serotonergic neurons (White et al., 1994); induce embryonic rat motor neuron survival (Hanson et al., 1998); and retinal ganglion cell responsiveness to trophic factors (Meyer-Franke et al., 1998).

The direct regulation of neuronal growth and differentiation in human adult olfactory neuroepithelial derived neural progenitors by RA, Shh, FN, and the PKA pathway has not been demonstrated. Thus, the purpose of this study was to investigate the roles of RA, FN, and Shh on NSFC neuronal lineage specification and differentiation *in vitro* using these adult human olfactory neuroepithelial progenitors. Here we report that the simultaneous treatment of NSFCs with RA and FN, or with RA and Shh can lead to neuronal morphology and transcription factor expression. Furthermore, multiple signal pathways are involved in the process of neuronal differentiation induced by these factors.

MATERIALS AND METHODS

Cell culture

The three different NSFC lines used in this study were obtained from adult olfactory neuroepithelium from a male (96 yr) cadaver (Roisen et al., 2001), and from a female (34 yr) and a male (38 yr) patient (Winstead et al., 2005) which were cultured as previously described (Roisen et al., 2001; Winstead et al., 2005). The NSFCs were adapted to the absence of serum via serial dilution of serum every 2 days

for a week until the cells were finally cultured in DFBNM (DMEM/F12 supplemented with 1% B27 and 0.5% N2) and 100 µg/ml gentamycin (GIBCO, Grand Island, NY) for one week (Zhang et al., 2004). Analyses of in vitro differentiation with the three lines (passages 10-20) were preformed. Since equivalent results were obtained with these three different lines, data from only one line has been presented.

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay

The viability of the NSFCs, plated at a density of 3×10^3 cells/well in 24-well plates (Falcon, Becton Dickinson, Franklin Lakes, NJ), after 7 days treatment with RA, FN, and/or Shh was measured with a MTT kit (Sigma, St. Louis, MO). Cells seeded in DFBNM without treatment served as controls. Mitochondrial dehydrogenases in living cells metabolized MTT into formazan crystals, the concentration of which was determined spectrophotometrically at a wavelength of 570 nm as described previously (Zhang et al., 2004).

Neurite formation

NSFCs were plated on glass coverslips in six well plates (3×10^4 cells/35 mm well) in DFBNM, and treated with various concentrations and combinations of RA, FN and Shh for 7 days {0.5 µM RA (RA0.5), 1 µM RA (RA1), 2 µM RA (RA2), 5 µM FN(FN), 15nM Shh (Shh), 1 µM RA and 5 µM FN (RA1FN5), 1 µM RA and 15nM Shh (RA1Shh). An alternate paradigm provided 4 days initial treatment with 1 µM RA and 5 µM FN, followed by 3 days of treatment with 1 µM RA and 15nM Shh (RA1FN5Shh)}. After treatment the neurite number, length, and neuritogenic index (neurite numbers X neurite lengths) were determined at 1-7 days in vitro (DIV). Cells

(500-1,000) were sampled systematically from standardized fields (total magnification 200X) with the aid of an eyepiece reticule under constant magnification with phase contrast optics. Only those primary neurites originating directly from the soma that were longer than the diameter of the cell body were evaluated in a double blind study (Roisen et al., 1981b).

Electron microscopy (EM)

The cultures treated with RA1FN5Shh (7 DIV) were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 at 4°C for 4 h. Following treatment with 1% osmium tetroxide, dehydration through an ethyl alcohol series and embedment, selected areas were mounted, sectioned, stained with 1% uranyl acetate and lead citrate. The neuritic spines were examined as previously described by our laboratory (Spoerri et al., 1990).

BrdU incorporation

The NSFCs (3×10^4 cells/well) were plated on 22 mm round glass coverslips in 6-well plates (Falcon, Franklin Lakes, NJ) and incubated at 37°C in 5% CO₂/95% air. To examine proliferation of NSFCs, 5-bromo-2'deoxyuridine (BrdU, 10 µM, Sigma) was added to the cells for 24 h before fixation. The cells were rinsed with cytoskeletal buffer (CB) twice and fixed in 3% paraformaldehyde in CB (10 min), when permeabilization was desired treated with 0.2% Triton X-100 (10 min, Sigma), and incubated in 0.6% H₂O₂ in Tris-Buffered Saline (TBS) for 30 min. Cells were incubated in 2 N HCl for 30 min at 37°C. Acid was removed by washing with TBS twice and neutralized with 0.1 M sodium borate (Sigma) for 10 min. Cells were incubated (1 h) in 3% bovine serum album (BSA) in TBS. Primary antibody anti-

BrdU was applied overnight (4°C). After washing (1 h) in TBS three times, the cells were incubated with secondary antibodies: Cy2-conjugated goat anti-mouse IgG (1:100, Cy2, Jackson Immunology Research Laboratories, West Grove, PA). Experiments were performed in triplicate; first and second antibody omission controls were performed with each experiment to ensure the specificity of staining.

Immunocytochemistry

The NSFCs (3×10^4 cells/well) were plated on 22 mm round glass coverslips in 6-well plates (Falcon) and incubated at 37°C in 5% CO₂/95% air for 24 h; treated with RA, FN, and Shh for 7 days prior to fixation for immunofluorescence. Cultures were incubated with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (1:1000, 2 mg/ml, Molecular Probes, Eugene, OR) for 30 min at 37°C for vital labeling of DNA when nuclear staining was desired. The coverslips were rinsed with CB twice and fixed in 3% paraformaldehyde in CB (10 min), when permeabilization was desired treated with 0.2% Triton X-100 (10 min, Sigma), and incubated (1 h) in 3% BSA in TBS. Primary antibodies (Table 7) were applied overnight (4°C). After washing (1 h) in TBS three times, the cells were incubated with secondary antibodies: Texas-red-conjugated goat anti-rabbit IgG, Texas-red-conjugated goat anti-mouse IgG, Cy2-conjugated goat anti-mouse IgG (all diluted 1:100, Cy2, Jackson Immunology Research Laboratories; Texas red, Molecular Probes). Experiments were performed in triplicate; first and second antibody omission controls were performed with each experiment to ensure the specificity of staining.

Coculture

Chicken skeletal muscle cells were removed from embryonic day 12 chicken pectoral muscles, dissociated with 0.25% trypsin at 37°C for 15 min and plated in 6-well plates (5×10^5 cells/well) with DF+10%FBS. From 2DIV, the cells were treated with cytosine β -D-arabinofuranoside (5 μ M; Sigma) to inhibit the growth of fibroblasts from the culture. After 7 DIV, cells were trypsinized (0.05% trypsin), and plated on glass coverslips in 6-well plates (5×10^5 cells/well) with DF+2%FBS for 3 days, and then cocultured with 7-day RA1FN5Shh treated NSFCs for another 7 days in DFBNM with RA1FN5Shh.

Protein kinase (PK) inhibitor treatments

NSFCs were plated in the flask (5×10^5 cells/flask) or 24-well plates (1×10^4 cells/well). Cells were preincubated with PKC inhibitor (GF109203X, 2.5 μ M), PKA inhibitor (H89, 2.5 μ M), MEK inhibitor (U0126, 2.5 μ M), PI 3-K inhibitor (Wortmannin, 0.5 μ M) for 30 min before the addition of culture medium with or without RA1FN5 for 48 h. All the inhibitors were obtained from Calbiochem, La Jolla, CA. Cell viability was determined with the MTT assay, and neurite numbers and length were determined as described above.

Western blot analysis

Proteins were separated on 10% -14% SDS-polyacrylamide gels and electroblotted onto nitrocellulose paper. Proteins were analyzed by Western blot assays performed as previously described (Zhang et al., 2005). Primary antibodies were listed in Table 7.

Statistical analysis

Statistical analysis (Graph pad Prism) was carried out using ANOVA (significance level $p < 0.05$). The mean and standard deviation of triplicate samples repeated a minimum of three times was determined for each of the three NSFCs lines. Since there were no detectable differences among the three cell lines, data has been reported without reference to which line was evaluated.

Table 7 Antibodies and specificity

Antibodies	Target	Source
Nestin, human monoclonal, 1:100	Neural stem cells	Chemicon International, Temecula, CA
Peripherin, polyclonal, 1:100	Neural crest cells	Chemicon
β -tubulin III, monoclonal, 1:100	Immature neurons and neurons	Sigma
α internexin, polyclonal, 1:100	Immature neurons and neurons	Chemicon
Tau, monoclonal, 1:100	Immature neurons and neurons	Chemicon
NF68, monoclonal, 1:400	Neurons	Sigma
NF160, monoclonal, 1:100	Neurons	Sigma
NF200, monoclonal, 1:100	Neurons	Sigma
VAcHT, polyclonal, 1:200, (WB) 1:1000	Cholinergic neurons	Chemicon
ChAT, monoclonal, 1:200, (WB) 1:1000	Cholinergic neurons	Chemicon
ACh, monoclonal, 1:200	Cholinergic neurons	Chemicon
TH, monoclonal, 1:200, (WB) 1:1000	Dopamine neurons	Sigma
Synapsin I, polyclonal, 1:500	Marker for synapse	Chemicon
NeuN, monoclonal, 1:100	Mature neurons	Chemicon
GFAP, monoclonal and polyclonal, 1:100	Astrocytes	Chemicon
OX42, monoclonal, 1:100	Microglia	Chemicon
GalC, monoclonal, 1:100	Immature oligodendrocyte	Chemicon
MBP, polyclonal, 1:200	Oligodendrocytes	Chemicon
T-CREB, rabbit monoclonal, 1:5000 (WB)	Transcription factor	Upstate, Lake Placid, NY
P-CREB (Ser133), monoclonal, 1:5000 (WB)	Transcription factor	Upstate
T-MAP kinase 1/2 (Erk1/2), polyclonal, 1:5000 (WB)	Protein kinase	Upstate
P-Erk1/2, polyclonal, 1:1000(WB)	Protein kinase	Upstate
HB9, monoclonal, 1:10	Transcription factor of Motoneurons	DSHB
Isl1/2, monoclonal, 1:10	Transcription factor of Motoneurons	DSHB
Actin, monoclonal, 1:2000 (WB)	Cell skeleton	Amersham Life Science

RESULTS

NSFC population in DFBNM

The NSFC population in DFBNM was heterogeneous. More than 97% of the cells were positive for β tubulin III and peripherin; $43.6 \pm 3.5\%$ for nestin; in the absence of triton; $25.4 \pm 1.9\%$ for A2B5; and $67.3 \pm 5.2\%$ for NCAM. In contrast, no cells were detected that were reactive for the astrocytic marker GFAP; microglial marker OX42; oligodendrocyte markers GalC or MBP; mature neuronal markers NeuN, HB9, Isl1/2, VACHT, ChAT, ACh, and TH (Data not shown). No differences in phenotypic expression were detected between the three lines selected for these studies.

RA1FN5Shh increased NSFCs neuritogenesis

To assess the effect of RA, FN, and Shh on neuritogenesis, the neurite number, length, and the derived neuritogenic index were determined over a 7 day treatment period. The treatments did not affect cell viability (Fig. 16A). The highest level of neuritogenic activity was produced by RA1FN5Shh (Fig. 16B-D, 16F); while neuritogenic activity in RA1FN5 or RA1Shh treatment were greater than in RA alone, which increased neurite number (Fig. 16B-D, 16E). Cells treated with RA1FN5Shh had more and longer neurites, which were covered with numerous spine-like processes (Fig. 16G).

NSFCs treated with RA, FN, and Shh expressed mature neuronal antigens

Following treatment with RA1FN5Shh for 7 days, 97% of NSFCs were β tubulin III⁺ and peripherin⁺ (Fig. 17A), $82 \pm 5.9\%$ Tau⁺ (Fig. 17B), and $86 \pm 7.4\%$ α internexin⁺ (Fig. 17C). In addition, NF68 ($31 \pm 3.2\%$, Fig. 17D) was localized in the

cell soma while NF160 ($27 \pm 2.4\%$, Fig. 17E) and NF200 ($24 \pm 2.1\%$, Fig. 17F) were expressed in the soma and neurites. In contrast, nestin expression was decreased to $17.2 \pm 1.9\%$, and no GFAP, OX42, GalC, and MBP was detected (Data not shown).

BrdU and NeuN were used to identify post mitotic neurons since mature neurons do not incorporate BrdU but express and therefore label with NeuN. RA or RA with FN and /or Shh after 7 days treatment significantly decreased the BrdU incorporation (Fig. 18E, 18F) and induced NeuN expression ($p < 0.01$, Fig. 18B, 18F). However FN, Shh or RA0.5 alone had no effect compared with the controls (Fig. 18A, 18F). Furthermore, the NeuN⁺ cells in cells treated with RA and FN was greater than that found in cells treated solely with RA ($p < 0.01$, Fig. 18F).

RA1FN5Shh not only induced NeuN expression, but also induced the expression of transcription factors of the motoneuron: HB9 (Fig. 18C, 18F) and Isl1/2 (Fig. 18D, 18F). Furthermore, RA combined with Shh induced more NSFCs to express motoneuron transcription factors than RA alone. However, RA0.5, FN, or Shh alone did not induce changes in neuronal lineage restriction. To determine if the motoneurons induced by RA1FN5Shh form functional connections with muscle fibers, the treated cells were cocultured with chicken skeletal muscle. Confocal imaging revealed co-localization at neuromuscular junctions of synapsin I and acetylcholine (Fig. 19A, 19C) or ChAT, which packages acetylcholine into vesicles for release at neuromuscular junctions (Fig. 19B).

The effect of protein kinase inhibitors on RA, FN, and Shh mediated expression of neuronal antigens and neurite formation by NSFCs

Recent studies have suggested that neurotrophic factors activate several protein kinase pathways (especially via CREB and Erk1/2) and produce neurite growth, neurogenesis, regeneration, and neuronal survival (Watt et al., 2004). We postulated that differentiation factors may activate the protein kinase pathways to produce changes of neuronal antigens and morphology similar to those of neurotrophic factors on olfactory-derived neurons.

As described above, cells treated with RA1FN5Shh not only underwent enhanced neurite formation, but also displayed numerous spines. Electron microscopic evaluation demonstrated the presence of vesicles within these spines (Fig. 16H) which were shown by immunocytochemistry to contain synapsin I (Fig. 20A) and VACHT (functional transporter for the neurotransmitter ACh, and ChAT (Fig. 20B-C). Western blot of cells following 7 day treatment of RA1FN5Shh confirmed the presence of these neurotransmitters (Fig. 20E-F). These neurotransmitters could be blocked by the treatments with various inhibitors: VACHT was partly blocked by PKC inhibitor GF109203X, MEK inhibitor U0126, and PI3-K inhibitor Wortmannin, but not by the PKA inhibitor H89. In contrast the increase of the ChAT was not blocked by any of the 4 inhibitors (Fig. 20E-F).

Furthermore, following 7-day treatment of RA1FN5Shh, some NSFCs ($11.6 \pm 1.5\%$) expressed the dopaminergic neuronal specific antigen, tyrosine hydroxylase (TH, Fig. 20D), which was confirmed by Western blot (Fig. 20E-F). The presence of TH could be partially blocked by application of GF109203X and U0126, but not by H89 and Wortmannin (Fig. 20E-F).

The involvement of PKC, PKA, PKB, and Erk pathways in NSFC RA and FN mediated neuritogenesis was investigated by 48 h exposure to specific inhibitors. After treatment with GF 109203X (PKC inhibitor) and /or H89 (PKA inhibitor), cell neuritogenesis was increased 61% (Length), and 44% (number)/cell (Fig. 21B-C, 21F). In contrast, after treatment with U0126 (MEK inhibitor) or Wortmannin (PI3-K inhibitor), cell neuritogenesis was decreased ($p < 0.01$, Fig. 21D-E) compared to RA1FN5 treated controls (Fig. 21A). There was no difference of viability among these groups. Similar results were obtained with RA1 and RA1Shh treated NSFCs following 48h exposure to the inhibitors (Data not shown).

More than one signal pathways effected neuritogenesis in olfactory derived NSFCs.

To test the role of RA, FN, and Shh on activation of CREB and Erk1/2 Western blot analysis using antibodies that specifically recognize the phosphorylated (active) forms of CREB and Erk1/2 was carried out. A significant increase of phosphorylated CREB (P-CREB) over the controls (basal levels) was observed at 1DIV-3 DIV when treated with Shh, but only after 3 DIV for FN. A significant decrease of P-CREB below the basal levels was noted at 7 DIV for Shh or FN. RA had no effect on the P-CREB in the NSFCs (Fig. 22A-B). After 1 day, an increase in phosphorylation of Erk1/2 over the controls was evident in RA-treated cultures, which although it decreased with time remained elevated above the basal levels after 7 DIV ($p < 0.01$; Fig. 22C-D). Shh and FN increased phosphorylation of Erk1/2 over the controls 3 DIV (Fig. 22C-D). To determine if the inhibition of neurite formation by U0126 and Wortmannin involved P-Erk1/2 or P-CREB, the inhibition of MEK, and PI3-K in the

NSFCs treated with RA, FN, or Shh for 2 days was examined by Western blot (Fig. 23A-F). The results demonstrated that U0126 significantly decreased P-Erk1/2 with Shh, FN, or RA treatment ($P < 0.01$; Fig. 23A, 23C), confirming that MEK played an important role in Erk1/2 activation of the NSFCs with Shh, FN, or RA treatment. Wortmannin also decreased P-Erk1/2 (Fig. 23A, 23D). Inhibition of MEK, decreased P-CREB induced by Shh (Fig. 23B, 23E). In contrast, inhibition of PI3-K had no effect on P-CREB (Fig. 23B, 23F).

Figure 16. Cell viability and neurite analysis. NSFCs (passage 10-20) were cultured in DFBNM alone (Control) or in medium supplemented as indicated for 7 days. (A) NSFC viability for equivalent cell numbers was determined by MTT; none of the treatments reduced the viability. (B) The greatest neurite number and (D) neuritogenic index was produced by the presence of RA1FN5Shh. (C) Longer processes were formed following treatment with RA1FN5 when compared to controls (E), NSFCs treated with RA1FN5Shh for 7 days increased numbers of neurites (F) and spine-like projections along their entire length compared to control cells (G). Ultrastructural examination of these projections revealed numerous vesicles (H). (E-G) Phase contrast microscopy. Data: mean \pm SD. * $p < 0.05$; ** $p < 0.01$. Each experiment included triplicate samples, all experiments were repeated a minimum of three times.

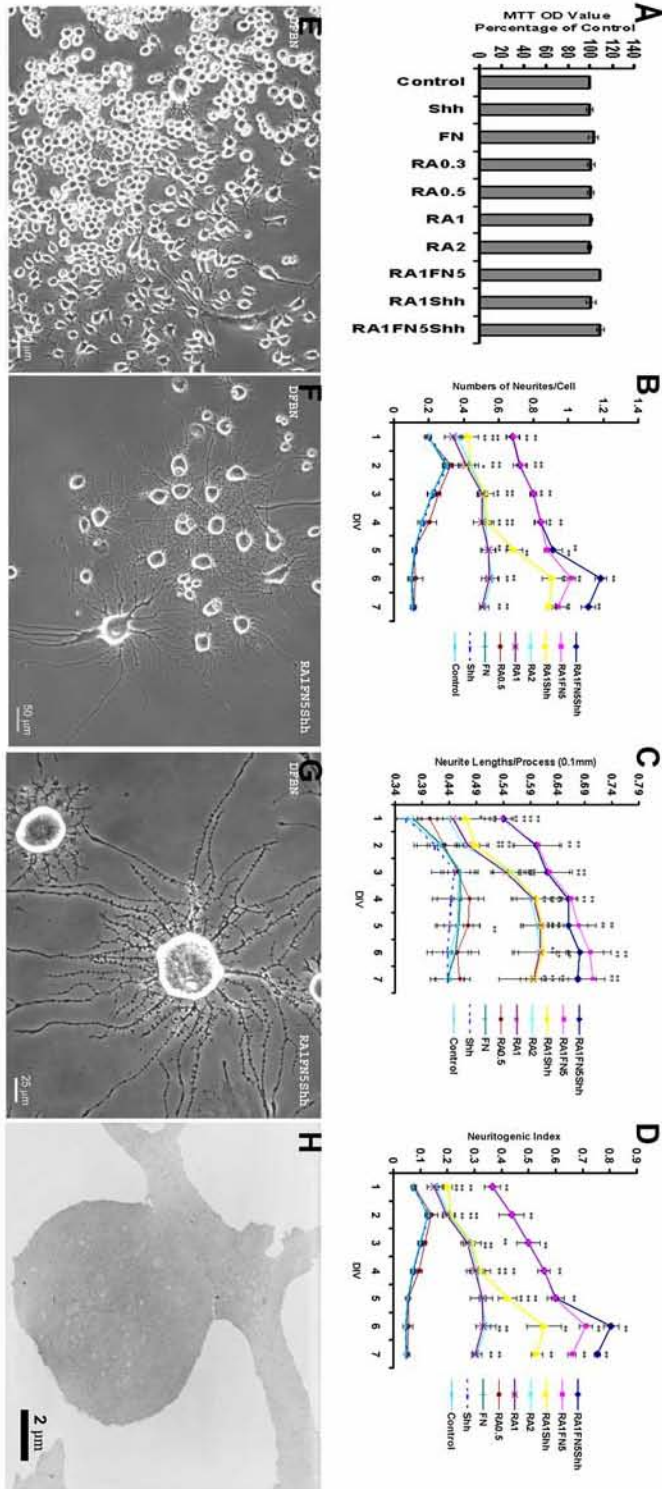


Figure 17. NSFCs expressed neuronal antigens following 7 days treatment with RA1FN5Shh: β tubulin III (A-C, green), peripherin (A, D-F, red), Tau (B, red), α -Internexin (C, red), NF68 within the soma (D, green), NF160 (E, green) and NF200 (F, green) within the soma and some neurites. DAPI (blue stain for DNA). Confocal images enhanced with DIC.

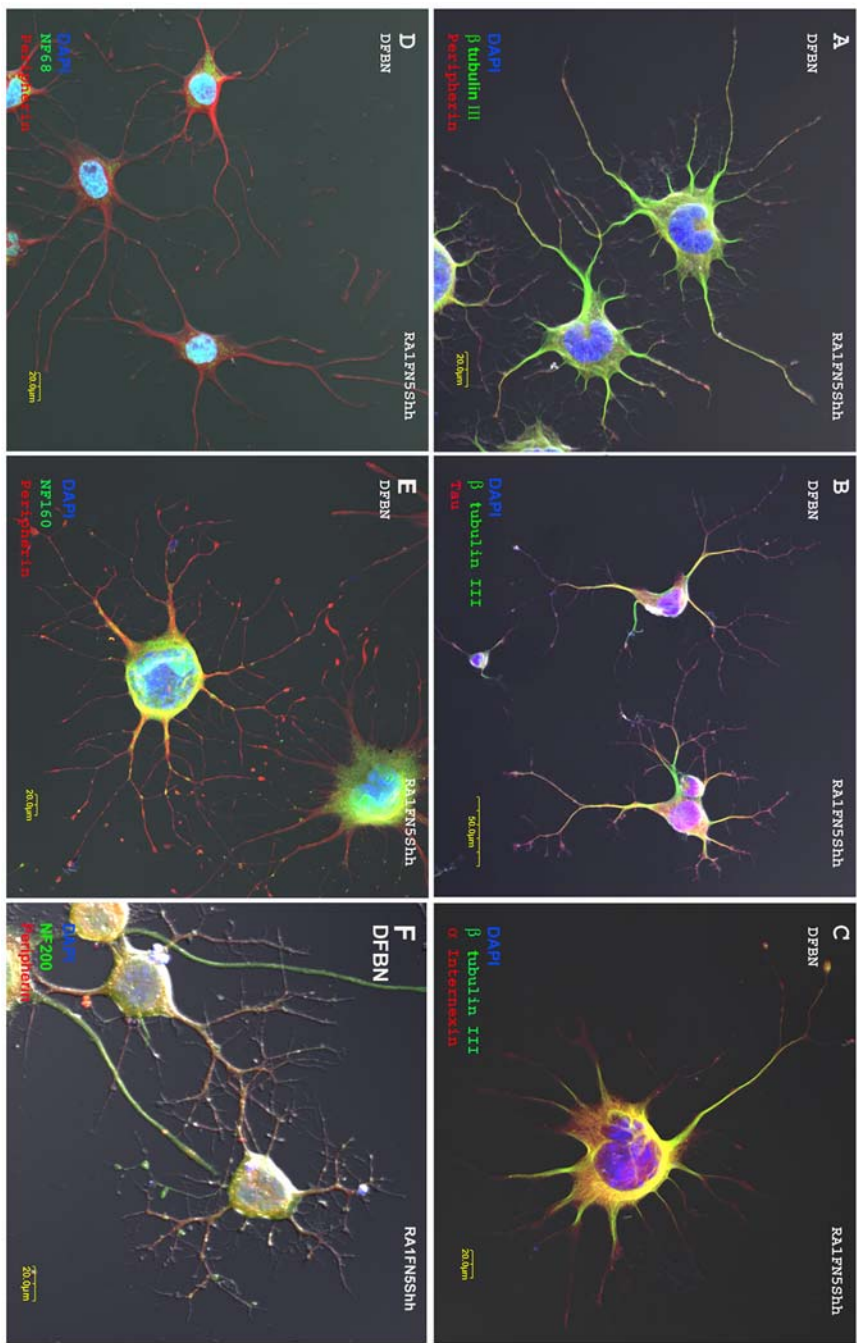


Figure 18. Neuronal phenotype and lineage changes. Adult human olfactory progenitors (passage 15) in DFBNM (controls) did not exhibit a neuron-like phenotype (A). After 7 days RA1FN5Shh treatment, NSFCs expressed peripherin (B-E, red), mature neuronal marker NeuN (B, green), the motoneuron markers HB9 (C, green) and Isl1/2 (D, green). Quantitative evaluation of treatments indicated that RA1FN5Shh increased neuronal restriction and decreased BrdU (E, green) incorporation ($p < 0.01$, E-F) compared with the controls (A). DAPI (blue stain for DNA). The confocal images (A-E) were enhanced with DIC. Data: mean \pm SD. * $p < 0.05$; ** $p < 0.01$.

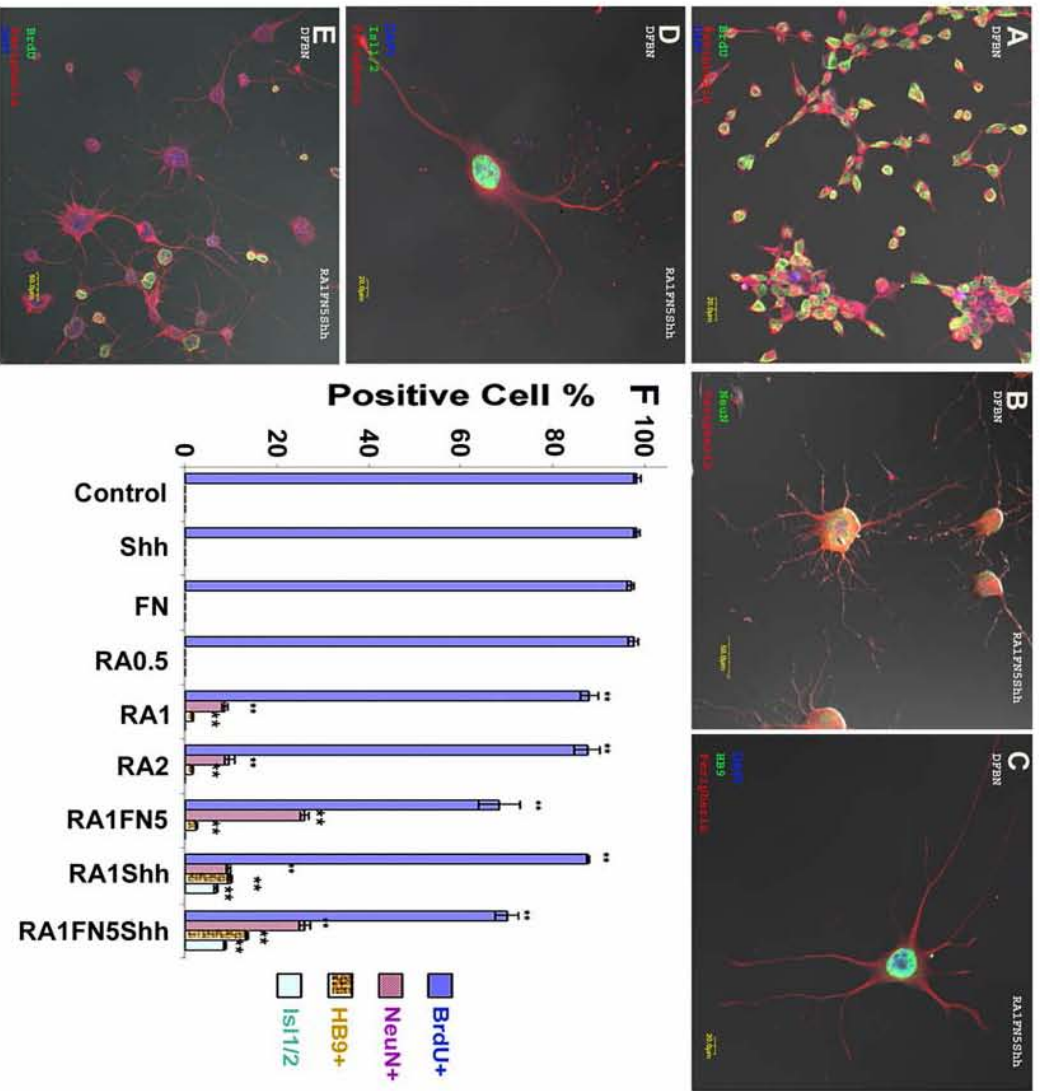


Figure 19. Coculture studies. Following 7-days RA1FN5Shh treatment NSFCs were cocultured with chick skeletal muscle for an additional 7 days during which they formed neuromuscular junctions; expressed ACh (A, C, green), synapsin I (A-C, red), and ChAT (B, green). DAPI (blue stain for DNA). The confocal images (A-C) were enhanced with DIC.

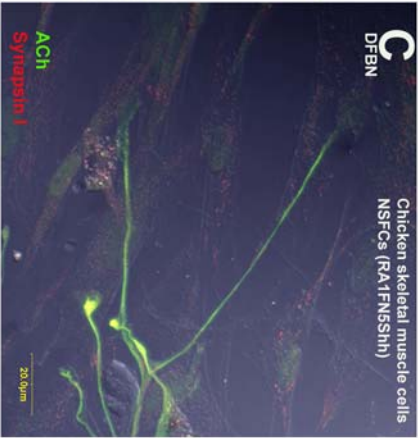
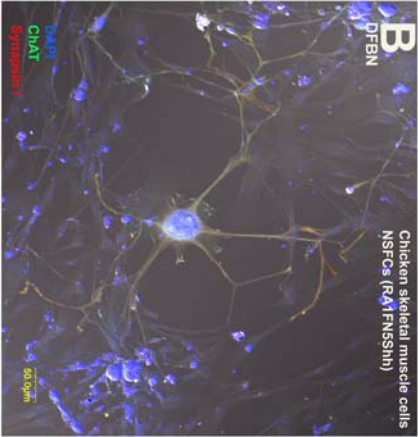
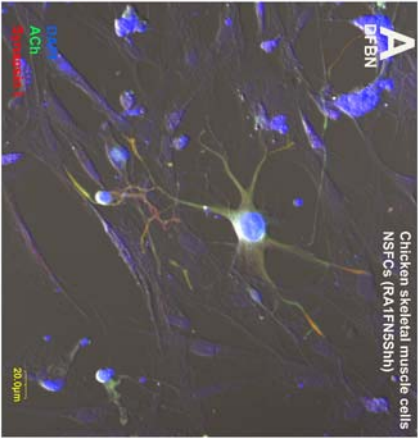


Figure 20. Following 7 days RA1FN5Shh treatment NSFCs had increased the expression of synapsin I (A, red), ChAT (B, green), VACht (C, red), and TH (D, green), they also expressed β tubulin III (A, C, green), peripherin (B, D, red). (E) Western blot analysis confirmed these results as protein bands ChAT (67 kDa), VACht (67-70 kDa), and TH (57/59 kDa) were detected. RA1FN5Shh treatment increased the expression of VACht, ChAT and TH compared with the controls ($p < 0.01$). (F) The expression of VACht was partly blocked by PKC inhibitor GF109203X, MEK inhibitor U0126, and PI3-K inhibitor Wortmannin, but not by the PKA inhibitor H89. In contrast ChAT was not blocked by any of the 4 inhibitors. The expression of TH was blocked by GF109203X and U0126 but not by H89 and Wortmannin. Quantification of protein bands expressed as means \pm SD. The density of the actin band was used as standard to adjust tracing quantification. NSFCs cultured in DFBNM as controls. The confocal images (A-D) were enhanced with DIC. DAPI (blue stain for DNA).

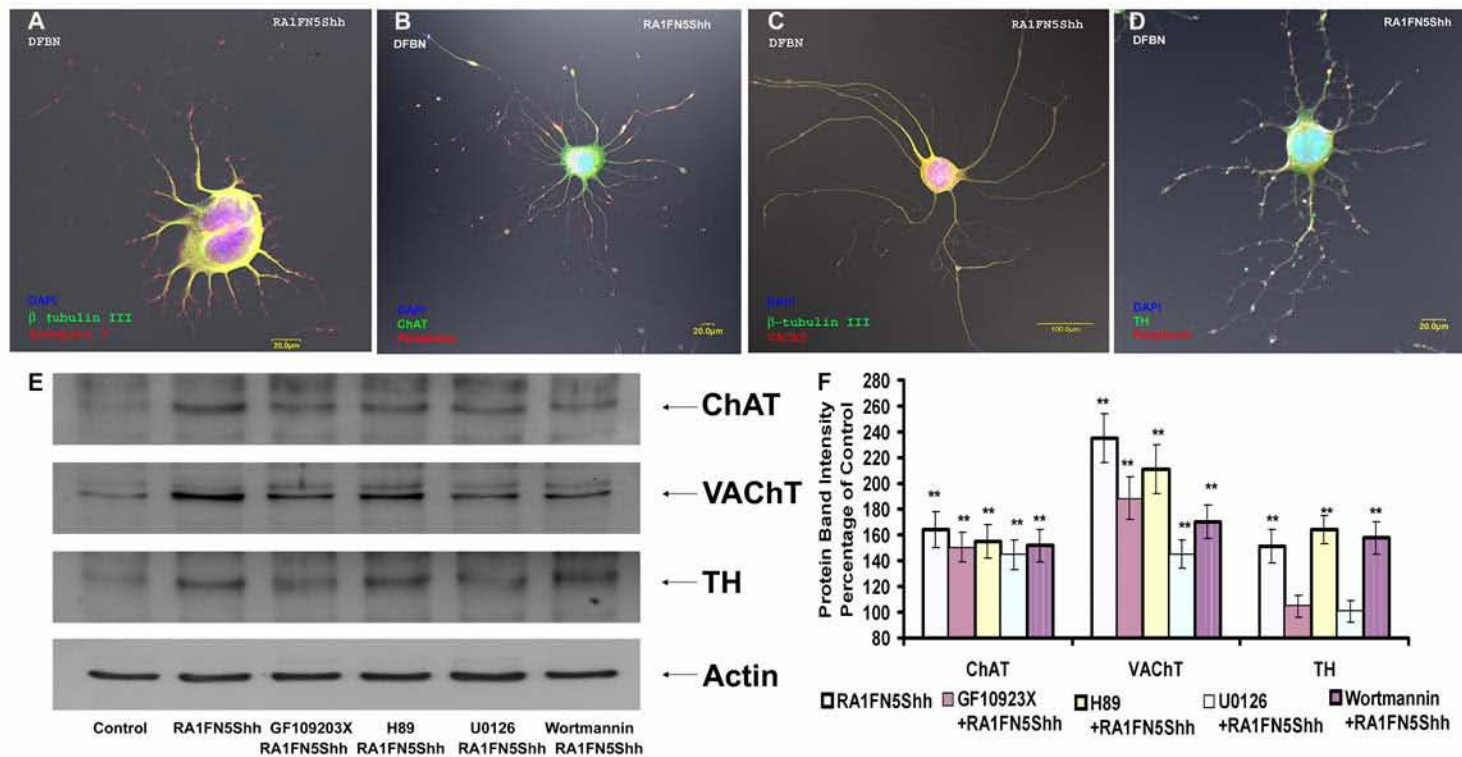


Figure 21. (A) Control NSFCs treated only with RA1FN5 in DFBNM. (B-F) PK inhibitors were employed to probe the mechanisms underlying neuronal restriction produced by RA1FN5. The cells were exposed simultaneously to the inhibitors for 30 min prior to incorporation of RA1FN5 into the medium, and evaluated after 48 h. (B, C, F) PKC and PKA inhibitors increased the number and length of neurites; while, (D, E) MEK and PI 3-K inhibitors decreased the number and length of neurites.

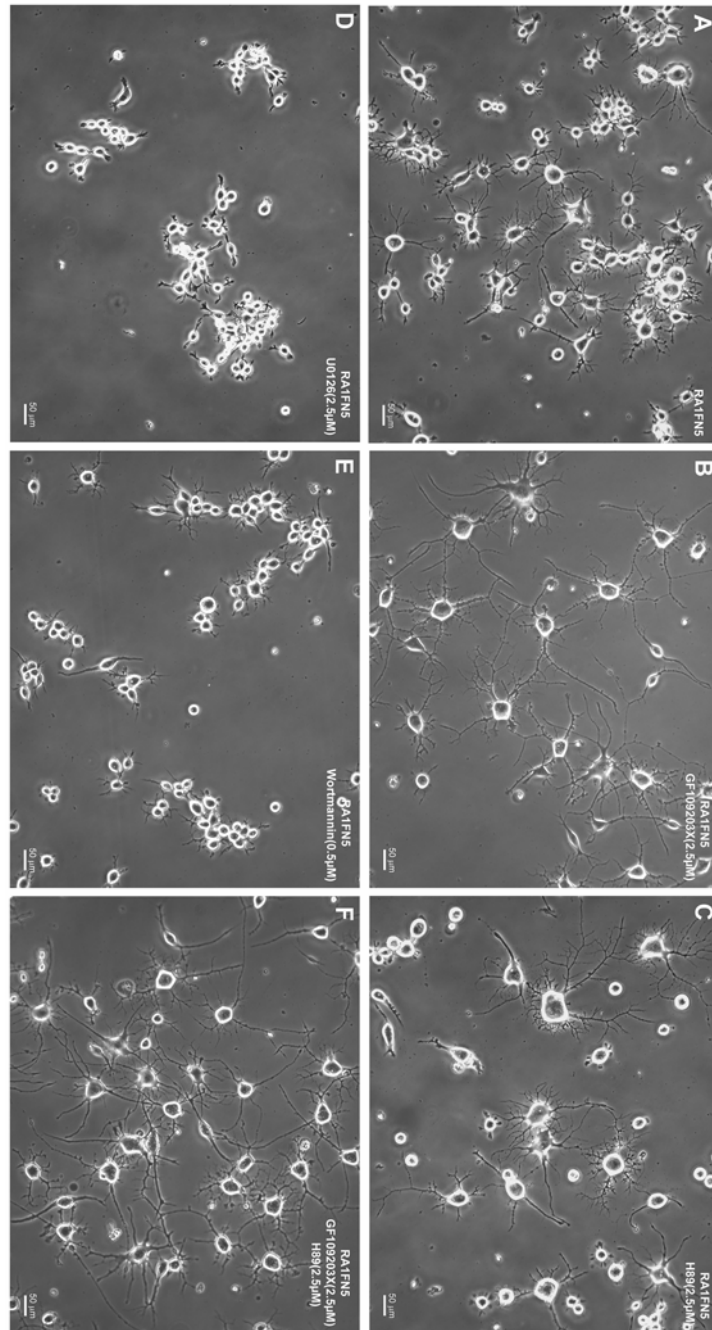


Figure 22. Western blot assay of expression of phosphorylated (P) CREB (46 kDa) and P-Erk1/2 (44/42 kDa) following 1-7 days treatment with RA (1 μ M), FN (5 μ M), or Shh (15 nM). The time expression of P-CREB (A) and P-Erk1/2 (C) is presented over time. The representative protein bands were analyzed densitometrically (B, D). These studies suggested that RA facilitated neuronal restriction by an Erk cell signaling pathway; in contrast, FN and Shh appeared to initially depend on a CREB mediated cascade followed by the Erk pathway. Total (T) Erk1/2 (44/42 kDa), and T-CREB (43 kDa) was used as controls for variation in cell density (there was no difference of T-Erk1/2 and T-CREB/ cell among the all groups according to the actin, data not shown). Quantification of protein bands expressed as mean \pm SD. * p <0.05; ** p <0.01 (compared to the controls, cultured in DFBNM).

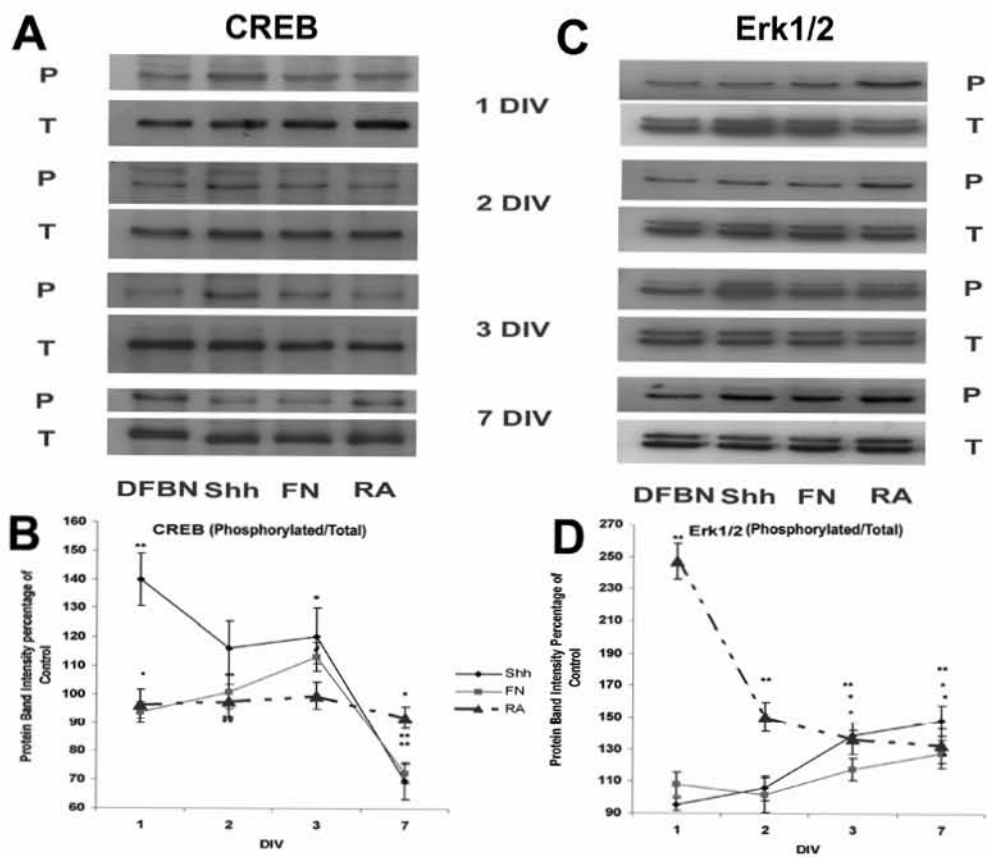
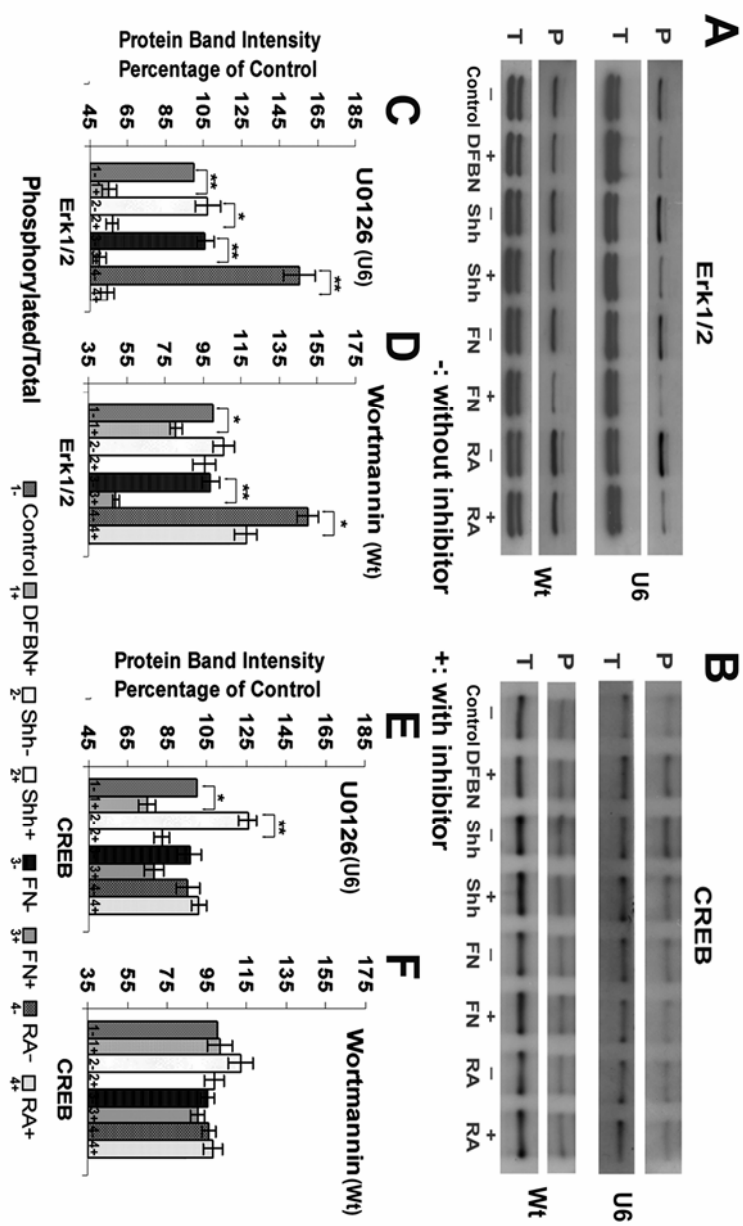


Figure 23. Western blot analysis followed by protein band densitometry demonstrated (A, C) inhibition of MEK by U0126 decreased P-Erk1/2; (A, D) inhibition of PI3-K by Wortmannin also decreased P-Erk1/2 (A, D); (B, E) Inhibition of MEK by U0126 decreased Shh induced P-CREB. In contrast, (B, F) inhibition of PI3-K by Wortmannin had no effect on P-CREB. Quantification of protein bands expressed as mean \pm SD. The density of the total T-Erk1/2 and T-CREB was used as standard to adjust tracing quantification. * $p < 0.05$; ** $p < 0.01$ (comparison of the presence and absence of inhibitor).



DISCUSSION

Neural stem cells have received much attention since they not only can be used in basic research on neurogenesis, but also have a therapeutic potential for various neurological diseases or injuries (Svendsen et al., 1996; McKay, 1997; Gage, 1998, Roisen et al., 2001). The human neural progenitors from adult olfactory neuroepithelium maintained in MEM10 or exposed to a variety of defined media and trophic factors were shown to remain relatively undifferentiated (Roisen et al., 2001; Zhang et al., 2004). These NSFCs appear to have an immature neuronal default, in which more than 97% cells expressed β tubulin III and peripherin. This suggests that the NSFCs obtained from adult human olfactory neuroepithelium may be different from embryonic and/or other species of neural stem cells (Chandran et al., 2004; Zhang et al., 2005). However, NSFCs have the characteristics of neural progenitors (Roisen et al., 2001; Winstead et al., 2005; Marshall et al., in press); they can be driven to differentiate into oligodendrocytes with transcription factors (Zhang et al., 2005). This study demonstrated that during treatment with RA, FN, and Shh, NSFCs gradually lost their progenitor characteristics such as nestin expression, and gained the properties of mature neurons.

Retinoic acid signal regulates neuronal differentiation in the developing nervous system (Zhang et al., 2003), in embryonic stem cells (Bibel et al., 2004), and adult neural progenitors (Hsieh et al., 2004). Shh signal plays a key role in generation of ventral neurons, in particular motoneurons (Wichterle et al., 2002) and dopamine neurons (Perrier et al., 2004). FN increases the cellular level of cAMP that is important for axonal elongation (Roisen et al., 1972a; 1972b). Here we show that

NSFCs can respond to these signals by expressing neuronal antigens including NF, NeuN, VACht, ChAT, and motoneuron transcription factors HB9 and Isl1/2 as well as exhibiting an elevated level of neuritogenesis. This is consistent with their roles as neural stem cells both *in vitro* and *vivo* (Diez del Corral et al., 2003; Roelink et al., 1995; Ericson et al., 1997). Furthermore, these signals induced a small percentage of NSFCs to express tyrosine hydroxylase (the rate-limiting enzyme for the synthesis of DA), thus suggesting the interesting possibility of their use as an autologous cell source for the treatment of Parkinson's disease. However, unlike the neural differentiation of ES cells, these responses could not be induced in NSFCs by FN or Shh alone. Furthermore, RA alone was unable to induce TH, and produced only limited expression of mature neuronal and motoneuronal antigens. In contrast, RA with FN, or RA with Shh treatment induced the highest numbers of cells with mature neuronal and motoneuronal antigens, as well as TH expressing cells. These results are consistent with the similar reports on embryonic chicken explants (Novitsch et al., 2003) and embryonic mouse stem cells (Wichterle et al., 2002). Since increased TH expression was blocked by PKC inhibitor GF109203X and MEK inhibitor U0126 but not by PKA inhibitor H89 and PI3-K inhibitor Wortmannin, it is likely that PKC and MAPK signaling cascades play essential roles in the RA, FN, and Shh-mediated differentiation of NSFCs into DA neurons.

It has been reported that RA induced neuronal differentiation of neuroblastoma cell line SH-SY5Y involved in phosphorylation of Erk1/2 (Singh et al., 2003). Consistent with this result, RA, or RA combined with FN/Shh stimulated neurite formation via Erk1/2 pathway in NSFCs; this response was blocked by MEK

inhibitor U0126 and PI 3-K inhibitor Wortmannin, indicating the important roles of the MAPK and PI3-K/PKB signaling cascades in NSFC neurite formation. It is also consistent with the similar results that BDNF induced both neuronal survival and outgrowth of sympathetic neurons of the rat embryonic superior cervical ganglia by activating the PI3-K/PKB and MEK/Erk signaling pathways (Atwal et al., 2000). In contrast, blocking PKC, or PKA significantly increased neurite formation of the NSFCs, which is different from the reported effects on hippocampal neurons (Lebrand et al., 2004) and Aplysia bag cell neurons (Kabir et al., 2001). However, similar effects have been observed previously, for example the inhibition by H89, a PKA inhibitor, promoted GM1 mediated neuroblastomal neuritogenesis (Singleton et al., 2000). In the PKA-deficient PC12 cells, Erk phosphorylation induced by RA returned to basal levels after 1 h of incubation, while CREB phosphorylation was sustained for 8 h (Canon et al., 2004). In NSFCs, RA induced Erk phosphorylation was sustained more than 7 days, in contrast increased P-CREB induced by FN or Shh returned below basal levels in 7 DIV, while P-Erk1/2 remained above basal levels after 3 DIV. Shh or FN alone did not induce neurite formation, indicating that Erk1/2 was perhaps activated by other factors induced by RA and thus resulted in neurite formation. RA could regulate gene expression for neurite formation by binding of RXR/RAR heterodimers to RA response elements (RAREs) (Canon et al., 2004). RA induced neuronal differentiation of SH-SY5Y cells also involved the activation of TGase and transamidation of RhoA (Singh et al., 2003). Furthermore, RA combined with FN/Shh increased neurite formation in NSFCs above the level produced by RA alone, suggesting that still unidentified kinases induced by FN and Shh enhanced

neurite formation. It has been reported that the rise in cAMP levels caused by FN stimulated neurite outgrowth in cultured neuroblastoma x glioma hybrid NG108-15 cells and embryonic rat hippocampal neurons via GTPases Rac and Cdc42, and PI3-K pathways (Leemhuis et al., 2004). It has also been reported that Shh induced motoneuron differentiation via the heterotrimeric G protein G12/ GTPase RhoA/Rhokinase pathway (Kasai et al., 2004). These results suggest that the Erk pathway combined with the RA conventional signaling pathway through binding of RXR/RAR heterodimers to RAREs play essential roles in neurite formation and perhaps are further enhanced by still unidentified kinases induced by FN and Shh treatment of NSFCs that results in neuritogenesis. Cell signaling pathways were also involved in neurotransmitter synthesis. VACHT was partly blocked by GF109203X and U0126, Wortmannin not by H89; the increase of ChAT was not blocked by the 4 inhibitors; indicating that the PKC, PI3-K/PKB, and MEK/Erk pathways influenced expression of VACHT in NSFCs. The precise regulatory mechanisms require further investigation.

Embryonic mouse stem cells treated with RA and Shh form motoneurons that have been reported to form functional synapses with target skeletal muscles *in vivo* (Wichterle et al., 2002). In this study, NSFCs induced by RA1FN5Shh exhibited altered neuronal morphology and antigenicity. The function of the resultant neurons was further investigated by coculture. NSFCs treated with RA1FN5Shh and cocultured with chicken skeletal muscles, appeared to form neuromuscular junctions. The spines on the end of neurites expressed synapsin I and contained vesicles. Furthermore, the expression of cholinergic neurotransmitters was identified in the

spines at the presumptive neuromuscular junctions. These results suggest that the neuronal differentiated NSFCs became functional neurons. Moreover they further suggest the possibility of their future potential in cell replacement strategies for the treatment of damaged motoneurons in spinal cord injury and amyotrophic lateral sclerosis.

In summary, these studies reveal that: (1) adult human olfactory-derived progenitors can be directed towards neuronal lineage by signal molecules RA, FN, and Shh which are known to control neuronal development in embryonic chick and rodent CNS. (2) In this model, RA and FN or Shh functioned cooperatively to produce neuronal differentiation. Neither FN nor Shh alone produced phenotypic changes. (3) RA with FN/Shh induced NSFC neurite formation that was blocked by U0126 and Wortmannin but not by GF109203X, and H89. (4) RA with FN/Shh treated NSFCs cocultured with chicken skeletal muscle, gained the capacity to form neuromuscular junctions

This is first demonstration that RA, FN, and Shh signals can direct adult human olfactory epithelial derived progenitors to differentiate into apparently functionally mature neurons. It also suggests that *in vitro* protocols may be employed to expand the therapeutic potential of these neural progenitors. Collectively these studies should heighten the interest in adult human olfactory neuroepithelial-derived progenitors as a readily accessible progenitor population which may provide an autologous source for cell replacement strategies for neurodegenerative diseases such as Parkinson's disease as well as for gene function and diagnostic studies.

ACKNOWLEDGMENTS

The authors thank George Harding for his assistance with confocal microscopy, Cathie Caple for technical assistance with electron microscopy and Michal Hetman for suggestions on the manuscript. This work was supported by NIH (1920RR15576 to F.J.R.), and Kentucky Spinal Cord Head Injury Research Trust (to M.Q.).

CHAPTER V

ROLE OF TRANSCRIPTION FACTORS IN THE MOTONEURON

DIFFERENTIATION OF ADULT HUMAN OLFACTORY

NEUROEPITHELIAL-DERIVED PROGENITORS

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Submitted: Stem Cells

INTRODUCTION

Olfactory neuroepithelium contains neural stem cells, which can regenerate and give rise to olfactory receptor neurons and supporting cells throughout the life (Moulton, 1974; Graziadei and Graziadei, 1979; Calof and Chikaraishi, 1989; Zhang et al., 2000). Stem cells from the olfactory neuroepithelium provide a unique source of adult neural progenitor cells, which can be obtained from a patient's olfactory neuroepithelium. Human olfactory neuroepithelium is located within the nasal cavity and can be obtained by nasal sinus surgery via endoscopic biopsy (Winstead et al., 2005). Therefore, it is possible to obtain these cells for autologous transplantation without highly invasive surgery. Previously, our lab has shown that cultures of olfactory epithelium can be grown to yield a population of neurosphere forming cells (NSFCs) (Roisen et al., 2001). Thus, they may have the potential to be used therapeutically to treat neurological disorders (Svendsen et al., 1996; McKay, 1997; Gage, 2000).

Basic-helix-loop-helix (bHLH) transcription factor Olig2 has been shown to be essential in the generation of oligodendrocytes and motoneurons *in vivo* (Novitch et al., 2001). Olig2 is expressed by motoneuron progenitors, has a key role in specifying pan-neuronal properties of developing motoneurons and precedes the expression of MNR2. Olig2 also directs the expression of motoneuron transcription factors including *Islet1/2* (*Isl1/2*), LIM-homeodomain (HD) gene and the *MNR2/HB9* homeobox gene in neural progenitor cells (Novitch et al., 2001). Isl1 and HB9 are defined markers for motoneurons and their progenitors (Mizuguchi et al., 2001). The

bHLH gene *Ngn2* is required for the generation of mouse motoneurons (Scardigli et al., 2001), the development of the mouse cranial sensory ganglia (Fode et al., 1998), and functions as a vertebrate neuronal lineage factor (Ma et al., 1996; Farah et al., 2000). The bHLH proteins Ngn2 and NeuroM coupled with Isl1 and Lhx3 specify motoneurons in the embryonic chicken spinal cord and mouse P19 embryonic carcinoma cells (Lee and Pfaff, 2003). Olig2 and Ngn2 were specifically coexpressed in motoneuron progenitor cells, and resulted in cell cycle exit and pan-neuronal properties of developing chicken motoneuron differentiation (Novitch et al., 2001; Mizuguchi et al., 2001). Lineage-restriction transcription factors HB9/MNR2 are specifically expressed in all somatic motoneurons. MNR2 is expressed in presumptive motoneuron progenitor cells and HB9 only in postmitotic motoneurons in the chick (Tanabe et al., 1998; Arber et al., 1999; Thaler et al., 1999). In contrast, HB9 is expressed in both motoneuron progenitors and postmitotic motoneurons in the mouse (Arber et al., 1999; Thaler et al., 1999; Odden et al., 2002; William et al., 2003). Transcription factor HB9 has been evolutionarily conserved, expressed selectively by embryonic motoneurons, and found essential for motoneuron development and differentiation (Lee and Pfaff, 2003). Coexpression of Ngn2 and MNR2 increased the number of Isl1/2 positive neurons in the chicken spinal cord compared with the MNR2 alone (Novitch et al., 2001). Mature motoneurons are characterized by their cytoskeletal components, ability to release neurotransmitters, surface receptors and their ability to form synapses (Reh, 2002; Song et al., 2002).

The signaling molecule retinoic acid (RA) has been reported to play important roles in neurogenesis (Sockanathan and Jessell, 1998; Maden, 2002; Novitch et al.,

2003; Diez del Corral et al., 2003; Bibel et al., 2004), neurite elongation (Corcoran and Maden, 1999) and synaptic plasticity (Chiang et al., 1998; Misner et al., 2001). Forskolin (FN) a molecule that increases intracellular cAMP, can stimulate axonal elongation (Roisen et al., 1972a; 1972b). Sonic hedgehog (Shh) regulates neuronal specification (such as motoneurons and dopaminergic neurons) in the ventral region of the embryonic CNS and can also direct human embryonic stem cells (Roelink et al., 1994; 1995; Ericson et al., 1997; Perrier et al., 2004). RA and Shh have been shown to determine the expression of HD and bHLH transcription factors which act on the neuronal fates, especially motoneurons in the ventral spinal cord and mouse embryonic stem cells as well (Novitsch et al., 2003; Diez del Corral et al., 2003; Wichterle et al., 2002; Sockanathan et al., 2003).

The role that these genes and modulatory molecules play in neuronal growth and differentiation of human adult-derived neural stem cells has not been demonstrated. Thus, the purpose of this study was to investigate the roles of *Olig2*, *Ng2*, and *HB9* genes in human neuronal lineage specification and differentiation *in vitro* using adult human olfactory neuroepithelial progenitors. Here we report that the simultaneous transfection of NSFCs with *Ng2-HB9* or *Olig2-HB9* combined with the treatment with RA, FN, and Shh can lead to the expression of motoneuron lineage restricted markers.

MATERIALS AND METHODS

Cell culture

The two different NSFC lines used in this study were obtained from adult olfactory neuroepithelium from a male (96 yr) cadaver (Roisen et al., 2001), and from

a female patient (34 yr) (Winstead et al., 2005). Frozen stock (passage 3-6) of each line was thawed rapidly and plated at a density of (5×10^5 cells/flask) in MEM with 10% heat-inactivated fetal bovine serum (FBS; GIBCO, Grand Island, NY), 100 μ g/ml gentamycin (MEM10) in flasks (25 cm², Corning Incorporated, Corning, NY) in humidified 5% CO₂/95% air (37°C) for 24 h. The NSFCs were adapted to the absence of serum via serial dilution of serum for a week until the cells were finally cultured in DFBNM (DMEM/F12 supplemented with 0.5% N2, and 1% B27) and 100 μ g/ml gentamycin for a week (Zhang et al., 2004; 2005), and then used for *in vitro* differentiation analyses between passages 10-20. Parallel experiments were preformed on NSFCs with both lines. Since equivalent results were obtained with these two different lines, data from only one line has been presented.

Construction of expression vectors

Full-length mouse *Olig2* cDNA, *Ngn2* and *HB9* were cloned into the pIRES2-EGFP expression vector (Clontech, Palo Alto, CA) individually (O-E, N-E, and H-E). For the *Olig2* and *Ngn2* co-expression vector, *Ngn2* cDNA was cloned into pIRES (Clontech) between NheI and EcoRI, and *Olig2* cDNA was inserted between XbaI and SalI. For co-expression of *Ngn2* and *HB9*, the *Ngn2* cDNA was cloned into the pLHCX expression vector (Clontech), and H-E was used (N-H). For co-expression of *Olig2* and *HB9*, the *Olig2* cDNA in pCAAGS vectors, and H-E was used (O-H). The pIRES2-EGFP and pIRES expression vectors served as controls (C-V). All expression vectors were verified by extensive DNA sequencing.

Transfection and selection

All plasmid constructs were introduced into the NSFCs by liposomal transfection. The cells were plated on glass coverslips in six well plates (3×10^4

cells/35 mm well) in DFBNM without antibiotics one day prior to transfection. NSFCs were transfected with each plasmid (4 µg/well) for 48 h according to the manufacture's protocol (Life Technologies, Rockville, MD). A further control was provided by lipofectamine alone. Two days following transfection, the cells were fixed or fed with DFB27M and selected with G418 (50 µg/ml; GIBCO, Grand Island, NY) (Zhang et al., 2005) or and hygromycin (50 µg/ml; GIBCO) for 7 days *in vitro* (DIV).

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay

The viability of the NSFCs after 2 days transfection and 7 days selection in flasks (25 cm², Corning Incorporated) was measured with MTT kit (Sigma St. Louis, MO). Cells were seeded in the flasks in DFBNM without transfection and selection as controls. Cells were plated at a density of 5×10^4 cells/well in 24-well plates (Falcon, B&D, Franklin Lakes, NJ) and MTT solution was added to each well. Mitochondrial dehydrogenases in living cells metabolized MTT into formazan crystals, the concentration of which was determined spectrophotometrically at a wavelength of 570 nm as described previously (Zhang et al., 2005).

Immunocytochemistry

The NSFCs (3×10^4 cells/well) were plated on 22 mm round glass coverslips in 6-well plates (Falcon) and incubated at 37°C in 5% CO₂/95% air for 24 h; transfected for 2 days; and either immediately fixed or selected for 7 days combine with 4 days treatment with 1 µM RA and 5 µM FN and another 3 days treatment with 1 µM RA and 15 nM Shh (RFS) prior to fixation for immunofluorescence. Cultures were incubated with 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (1:1000, 2

mg/ml, Molecular Probes, Eugene, OR) for 30 min at 37°C for vital labeling of DNA when nuclear staining was desired. The coverslips were rinsed with cytoskeletal buffer (CB) twice and fixed in 3% paraformaldehyde in CB (10 min), when permeabilization was desired treated with 0.2% Triton X-100 (10 min, Sigma), and incubated (1 h) in 3% bovine serum album (BSA) in Tris-Buffered Saline (TBS). Primary antibodies (Table 8) were applied overnight (4°C). After washing (1 h) in TBS three times, the cells were incubated with secondary antibodies: Texas-red-conjugated goat anti-rabbit IgG, Texas-red-conjugated goat anti-mouse IgG, Cy2-conjugated goat anti-mouse IgG (all diluted 1:100, Cy2, Jackson Immunology Research Laboratories, West Grove, PA; Texas red, Molecular Probes, Eugene, OR). Experiments were performed in triplicate; first and second antibody omission controls were performed with each experiment to ensure the specificity of staining (Zhang et al., 2004).

Neurite formation

NSFCs were plated on glass coverslips in six well plates (3×10^4 cells/35 mm well) in DFBNM. After transfection and selection combined with the treatment of RFS for 7 DIV, the number and length of neurites were determined. Cells (500-1,000) were sampled systematically from standardized fields (total magnification 200X) with the aid of an eyepiece reticule under constant magnification with phase contrast optics. Only those primary neurites originating directly from the soma of the NSFCs that were greater than the diameter of the cell body were evaluated in a double blind study (Roisen et al., 1981b; Zhang et al., 2005).

Western blot analysis

Western blot analysis was employed to support the immunofluorescence studies. Proteins from NSFCs cultured in DFBNM without selection, NSFCs transfected with control vectors, as well as NSFCs transfected with the vectors plus each combination of transcriptions factors (*Olig2*, *Ngn2*, *HB9*, *Olig2-Ngn2*, *Ngn2-HB9*, and *Olig2-HB9*), selected and combined with RFS treatment in all groups, were collected in lysis buffer. After 10 min incubation (4°C), samples were centrifuged at 12,000 x g (20 min) and the protein concentration of each supernatant was determined. The protein samples (20 µg/well) were electrophoresed on 10% -14% SDS-polyacrylamide gels along with standardized molecular size marker proteins in an adjacent lane and transferred from gel to nitrocellulose paper. Nonspecific binding was blocked (1 h) with 5% nonfat dry milk in TBST buffer. Blots were incubated (4°C overnight) following addition of primary antibodies (Table 8). Blots were washed with TBST buffer four times for 20 min and then three times for 10 min. Washed blots were incubated (1 h) with polyclonal horseradish peroxidase-labeled anti-rabbit IgG (1:1000) as well as monoclonal horseradish peroxidase-labeled anti-mouse IgG (1:1000). Chemiluminescence Western blotting detection (Bio-Rad, Hercules, CA) was employed to identify bound antibodies. Densitometry of the protein bands was carried out on a Molecular Dynamics gel scanner (Molecular Dynamics, Sunnyvale, CA). Data were analyzed using the Image Quant software programs supplied by the manufacturer.

BrdU (5-bromo-2'deoxyuridine) incorporation

The NSFCs (3×10^4 cells/well) were plated on 22 mm round glass coverslips in 6-well plates (Falcon, Franklin Lakes, NJ) and incubated at 37°C in 5% CO₂/95% air. To examine mitotic activity of NSFCs after transfection and selection combined with RFS treatment, 5-bromo-2'deoxyuridine (BrdU, 10 µM, Sigma) was added to the cells for 24 h before fixation. The cells were rinsed with cytoskeletal buffer twice and fixed in 3% paraformaldehyde in CB (10 min), when permeabilization was desired treated with 0.2% Triton X-100 (10 min, Sigma), and incubated in 0.6% H₂O₂ in Tris-Buffered Saline (TBS) for 30 min. Cells were incubated in 2 N HCl for 30 min at 37°C. Acid was removed by washing with TBS twice and neutralized with 0.1 M sodium borate (Sigma) for 10 min. Cells were incubated (1 h) in 3% BSA in TBS. Primary antibody anti-BrdU was applied overnight (4°C). After washing (1 h) in TBS three times, the cells were incubated with secondary antibodies: Cy2-conjugated goat anti-mouse IgG, or Texas-red-conjugated goat anti-mouse IgG (1:100, Cy2, Jackson Immunology Research Laboratories). Experiments were performed in triplicate; first and second antibody omission controls were performed with each experiment to ensure the specificity of staining.

Co-culture

Chicken skeletal pectoral muscles were removed from 12 day embryos and dissociated with 0.25% trypsin at 37°C for 15 min and plated in 6-well plates (5×10^5 cells/well) with DF+10% FBS. From 2DIV, the cells were treated with cytosine β-D-arabinofuranoside (5 µM; Sigma) to eliminate dividing cells and therefore substantially reduce the presence of fibroblasts. After 7 DIV, cells were trypsinized

(0.05% trypsin in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS), and plated on glass coverslips in 6-well plates with DF+2%FBS for 3 days. NSFCs were transfected with *Ngn2-HB9*, or *Olig2-HB9* and treated with 1 μM RA and 5 μM FN, and selection for 4 days (RF). The established muscle straps were co-cultured with these NSFCs in DFBNM supplemented with 1 μM RA and 15 nM Shh for an additional 3 days in DFBNM (RS).

Statistics

Statistical analysis (Graph pad Prism) was carried out using ANOVA (significance level $p < 0.05$). Cells (500-1,000) were sampled systematically from standardized fields (total magnification 200X) of cells stained for each marker. The mean and standard deviation of triplicate samples repeated a minimum of three times was determined for each of the two NSFCs lines. Since there were no detectable differences between the two cell lines, data has been reported without reference to which line was evaluated.

Table 8 Antibodies and specificity

Antibodies	Target	Source
Nestin, human monoclonal, 1:100	Neural stem cells	Chemicon International, Temecula, CA
β -tubulin III, monoclonal, 1:100	Immature neurons and neurons	Sigma
NF68, monoclonal, 1:400	Neurons	Sigma
NF160, monoclonal, 1:100	Neurons	Sigma
NF200, monoclonal, 1:100	Neurons	Sigma
VAcHT, polyclonal, 1:200, (WB) 1:1000	Cholinergic neurons	Chemicon
ChAT, monoclonal, 1:200, (WB) 1:1000	Cholinergic neurons	Chemicon
ACh, monoclonal, 1:200	Cholinergic neurons	Chemicon
TH, monoclonal, 1:200, (WB) 1:1000	Dopamine neurons	Sigma
Synapsin I, polyclonal, 1:500	Marker for synapse	Chemicon
NeuN, monoclonal, 1:100	Mature neurons	Chemicon
GFAP, monoclonal and polyclonal, 1:100	Astrocytes	Chemicon
OX42, monoclonal, 1:100	Microglia	Chemicon
GalC, monoclonal, 1:100	Immature oligodendrocyte	Chemicon
MBP, polyclonal, 1:200	Oligodendrocytes	Chemicon
Olig2, polyclonal, 1:1000(WB)	Transcription factor	Drs. David H. Rowitch and Charles D. Stiles
Ngn2, polyclonal, 1:1000(WB)	Transcription factor	Chemicon
HB9, monoclonal, 1:10; 1:1000(WB)	Transcription factor of Motoneurons	DSHB
Isl1/2, monoclonal, 1:10	Transcription factor of Motoneurons	DSHB
Actin, monoclonal, 1:2000 (WB)	Cell skeleton	Amersham Life Science

RESULTS

NSFC population

The heterogeneous nature of the NSFC population just prior to transfection was demonstrated by immunolocalization of fixed triton-treated cells. More than 97% of the cells were β tubulin III+ and peripherin+; $43.6 \pm 3.5\%$ nestin+; in the absence of triton; $25.4 \pm 1.9\%$ A2B5+; and $67.3 \pm 5.2\%$ NCAM+ prior to transfection in DFBNM. In contrast, no cells were detected that were reactive for galactosylceramide (GalC), myelin basic protein (MBP) an oligodendrocyte marker, glial fibrillary acidic protein, (GFAP) an astrocyte marker, OX42 a microglia marker, a mature neuron marker (NeuN), vesicular acetylcholine transporter (VACHT), choline acetyltransferase (ChAT), acetylcholine (ACh), tyrosine hydroxylase, the rate-limiting enzyme for the synthesis of dopamine (TH), Olig2, Ngn2, HB9, or Isl1/2 (Data not shown). No differences in phenotypic expression were detected between cells selected from the two different lines employed in these studies.

NSFCs transfected with *Ngn2-HB9* had increased expression of mature neuronal and motoneuronal markers and elevated neuritogenic activity

To examine the phenotypic expression of NSFCs following transfection and selection, representative cultures and controls were compared. Non-transfected NSFCs or those transfected with lipofectamine alone died within one week following selection with 50 μ g/ml G418 or/ and 50 μ g/ml hygromycin. Furthermore, there was no difference in cell viability between the various experimental groups as determined by the MTT assay (Fig. 31). Transfection with control vectors, single genes, or *Olig2-Ngn2* combined with RFS treatment resulted in no morphologic changes compared

with RFS treatment alone or as indicated for, *Olig2-EGFP*, *Ngn2-EGFP*, *HB9-EGFP* alone, or *Olig2-Ngn2* (Fig. 24 A-F, Fig. 25). In contrast, the morphology of NSFCs transfected with both *Ngn2* and *HB9* and selection combined with RFS treatment for 7 days underwent dramatic changes that resulted in a phenotype characteristic of neurons (Fig. 25A-B). Immunofluorescent analysis demonstrated that NSFC transfected with *Ngn2-HB9* combined with the RFS treatment, not only had expression rates of more than 97% for each of the following: Ngn2 (Fig. 26A), HB9 (Fig. 26B), β tubulin III (Fig. 26C) and peripherin (data not shown). Increased expression was also noted for neuronal markers: NF68 (Fig. 26D), NF160 (Fig. 26E), NF200 (Fig. 26F), NeuN (Fig. 25A, Fig. 27A), and ChAT (Fig. 27B), and the motoneuronal transcription factor Isl1/2 (Fig. 25A, Fig. 27C). In contrast, the expression of nestin and BrdU labeling were decreased (Fig. 25B, Fig. 27D-E); no markers for other cell lineages were detected including GalC (data not shown), MBP, GFAP, or OX42 in the transfected cells (Fig. 27F-H).

Furthermore, the neurite lengths and numbers of NSFCs were increased following transfection, selection, and treatment with RFS as compared to cells exposed to RFS without transfection ($p < 0.01$) (Fig. 32).

Western blot analysis of the effects of transcription factors *Olig2*, *Ngn2*, and *HB9* on the neuronal lineage-restriction of NSFCs

Though no *Olig2*⁺ cells were detected by immunohistochemistry, Western blot results indicated the presence of a low level of endogenous *Olig2* expression in the groups without transfection with *Olig2* (Fig. 28A-B). However, the expression of *Olig2* was much higher after transfection with *Olig2* (Fig. 28A-B). Similarly, the

expression of *Ngn2* and *HB9* were detected after transfection with *Ngn2* and *HB9* respectively (Fig. 28A-B). The neuronal phenotype induced by the transcription factors *Ngn2*-*HB9* was further confirmed by Western blot analysis. In NSFCs cultured in defined medium (DFBNM, controls) or transfected by control vectors, *Olig2-EGFP*, *Ngn2-EGFP*, *HB9-EGFP* alone or *Olig2-Ngn2* with the RFS treatment, VAcHT, ChAT and TH were not increased compared to cells exposed solely to RFS (Fig. 29A-B). However, in the cells co-transfected with both *Ngn2* and *HB9* with the RFS treatment, the expression was increased ($p<0.01$, Fig. 29A-B).

Olig2* can mimic the effects of *Ngn2* in inducing neuronal phenotype in collaboration with *HB9

Despite the low level of expression of *Olig2*, there was a strong induction of *Ngn2* expression following transfection with *Olig2* (Fig. 28A-B). Since a high level expression of *Ngn2* in collaboration with *HB9* could induce increased expression of neuronal antigens and neurite formation of NSFCs when combined with the treatment of RFS, *Olig2* and *HB9* cDNAs were introduced into NSFCs to determine if they could mimic the effects of *Ngn2*-*HB9*. Immunohistochemistry and Western blot results confirmed the expression of *Olig2* and *HB9* after transfection with *Olig2*-*HB9* (Fig. 28A-B, Fig. 30A-B). Co-transfection with *Olig2*-*HB9* in combination with the RFS treatment induced the expression of neuronal markers NeuN, VAcHT, ChAT, TH and the motoneuronal marker *Isl1/2* (Fig. 25A-B, Fig. 29A-B, Fig. 30C), as well as increased neurite lengths ($p<0.05$) and numbers ($p<0.01$) (Fig. 32A-B). In contrast, the expression of nestin and BrdU labeling were decreased (Fig. 25A-B).

Cocultured of NSFCs transfected with *Ngn2-HB9* or *Olig2-HB9* with Chicken skeletal muscle

A few axonal-muscular contacts were observed when RF treated non-transfected NSFCs or NSFCs transfected with control vectors in DFBNM were plated on an established layer of chicken skeletal muscle (controls) for 3 days with RS treatment. In contrast, NSFCs transfected with *Ngn2-HB9* or *Olig2-HB9* after 4 day selection and treatment with 1 μ M RA and 5 μ M FN (RF), cocultured with muscle for 3 days in the presence of 1 μ M RA and 15 nM Shh (RS), formed multiple processes which often were observed in direct contact with the muscle cells (Fig. 30D-H). As demonstrated with confocal microscopy, neurites of NSFCs and muscle cells formed apparent neuromuscular junctions, in which synapsin I (Fig. 30D, 30H), ChAT (Fig. 30E) or acetylcholine (Fig. 30F, 30G) were co-localized.

Figure 24. NSFCs (passage 10-20) transfected with either single genes alone or *Olig2-Ngn2* did not exhibit morphological changes. (A) Immunolocalization demonstrated that GFP (green, A-D) was expressed in the NSFCs after transfection with *C-V*, *O-E*, *N-E*, *H-E* and selection combined with the treatment with RFS. After transfection with *O-E*, *N-E*, *H-E* and *O-N* for 2 days and selection for 7 days with RFS treatment, NSFCs appeared similar to non-transfected controls and expressed the transfected cDNA: Olig2 (red, B, E), Ngn2 (red, C, F), and HB9 (green, D). DAPI (blue stain for DNA). (Confocal microscopy, A-F, DIC).

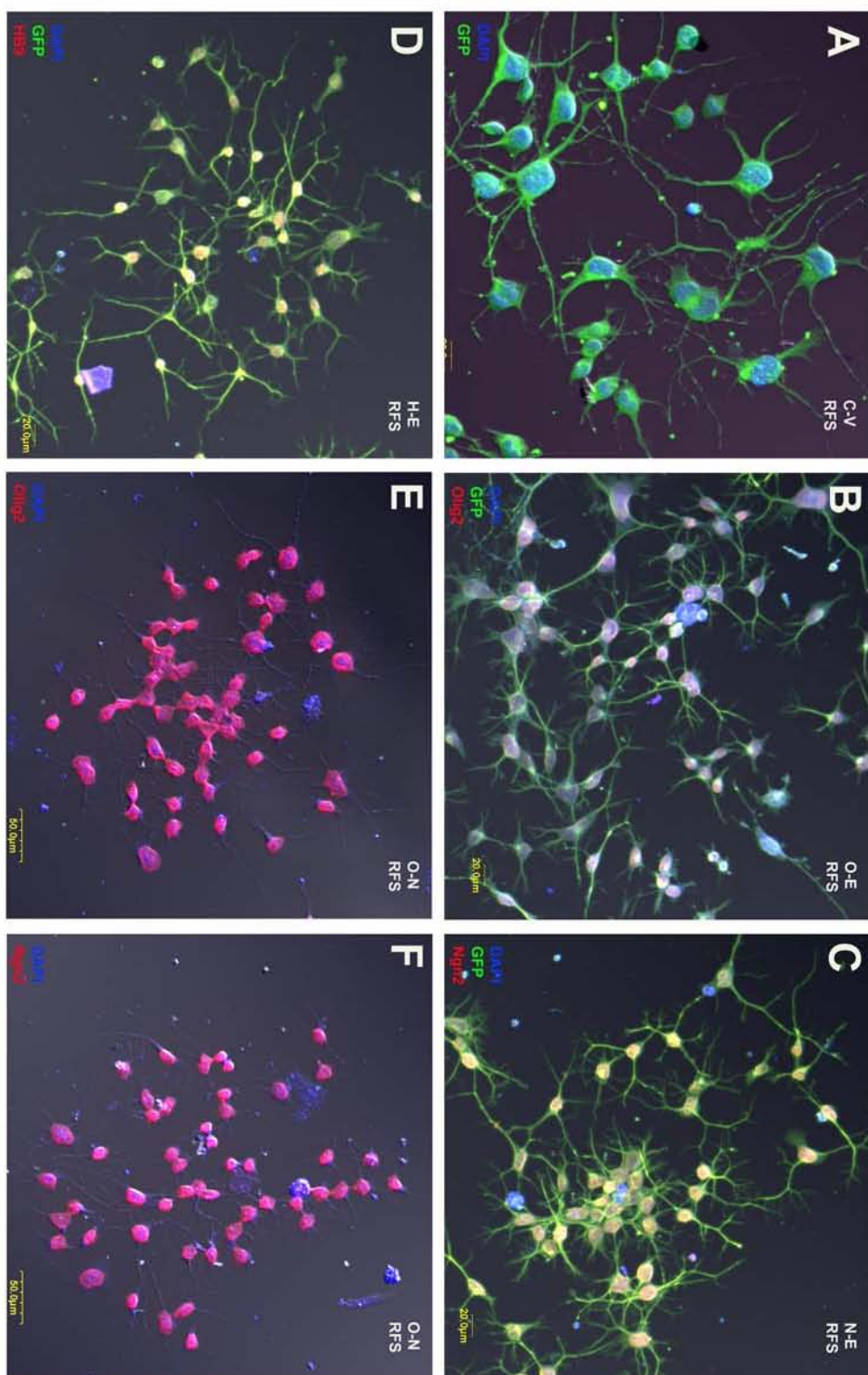


Figure 25. Immunocytochemical analysis of transfected NSFCs (passage 10 -20, cultured in DFBNM) transfected with control vector (C-V), *Olig2-EGFP* (O-E), *Ngn2-EGFP* (N-E), *HB9-EGFP* (H-E), *Olig2-Ngn2* (O-N), *Ngn2-HB9* (N-H), and *Olig2-HB9* (O-H) for 2 days after 7 days of selection and with or without RFS treatment was performed after fixation. NSFCs cultured 9 days in DFBNM without transfection served as the control. After transfection with N-H or O-H, and selection combined with RFS treatment, NSFCs expression of neuronal and motoneuronal antigens NeuN and Isl1/2 increased (A), while the expression of nestin and incorporation of BrdU (B) compared with RFS (**, $p < 0.01$, t-test). Values are means expressed as percentages \pm standard deviation (n = 54 fields). Each experiment employed triplicate samples and was repeated a minimum of three times.

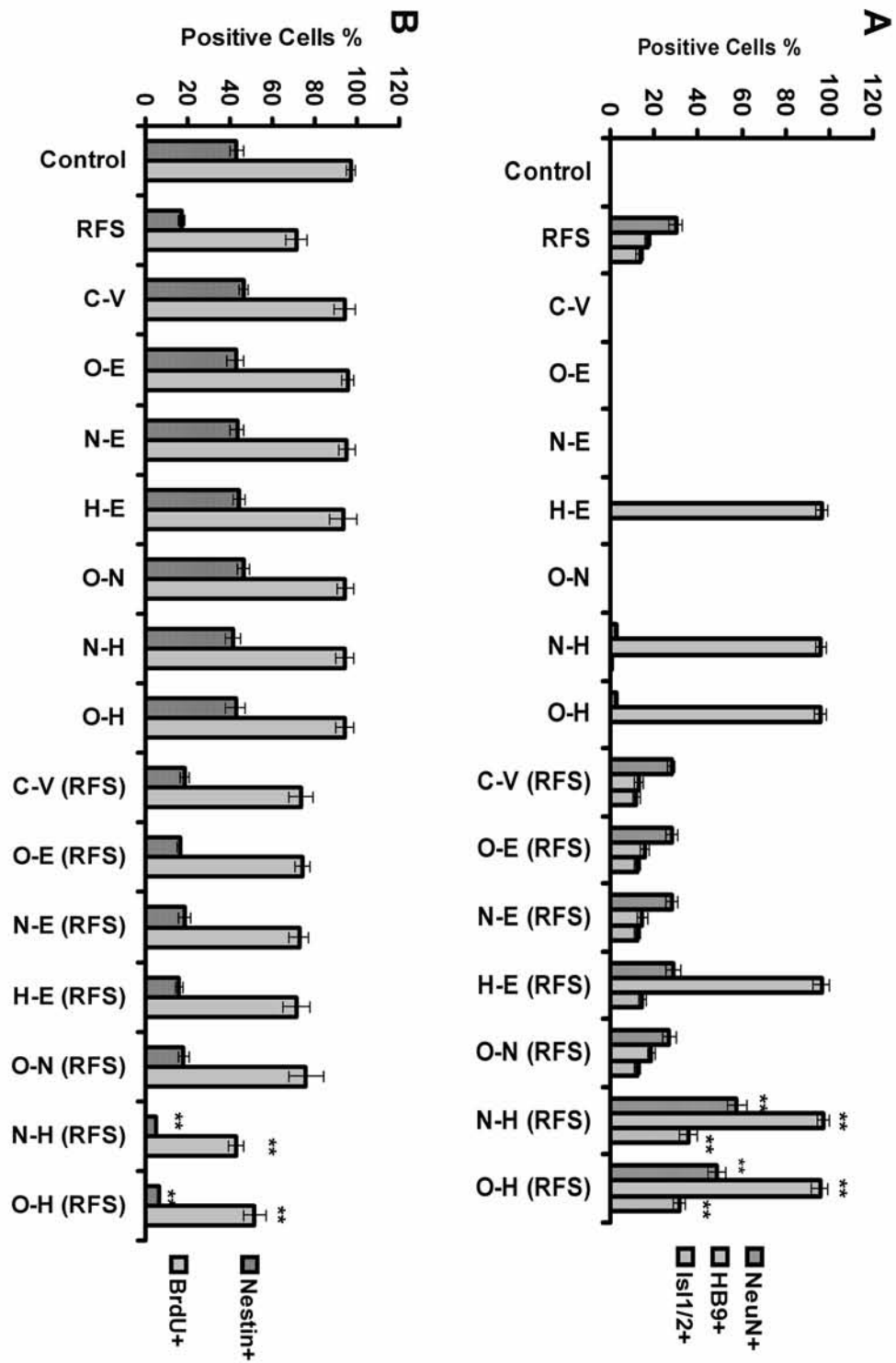


Figure 26. Following 2 days co-transfection with *Ngn2-HB9* and 7 days selection with RFS treatment, NSFCs (passage 10-20) exhibited a characteristic neuronal phenotypic expression. These representative micrographs illustrate immunolocalization with (A-F) GFP (green) in *HB9* vectors and transfected cDNA: (A) *Ngn2* (red), (B) *HB9* (red) as well as for several neuronal specific lineage restricted antigens (C) β tubulin III (red), (D) NF68 (red), (E) NF160 (red), and (F) NF200 (red). DAPI (blue stain for DNA). Confocal microscopy with DIC.

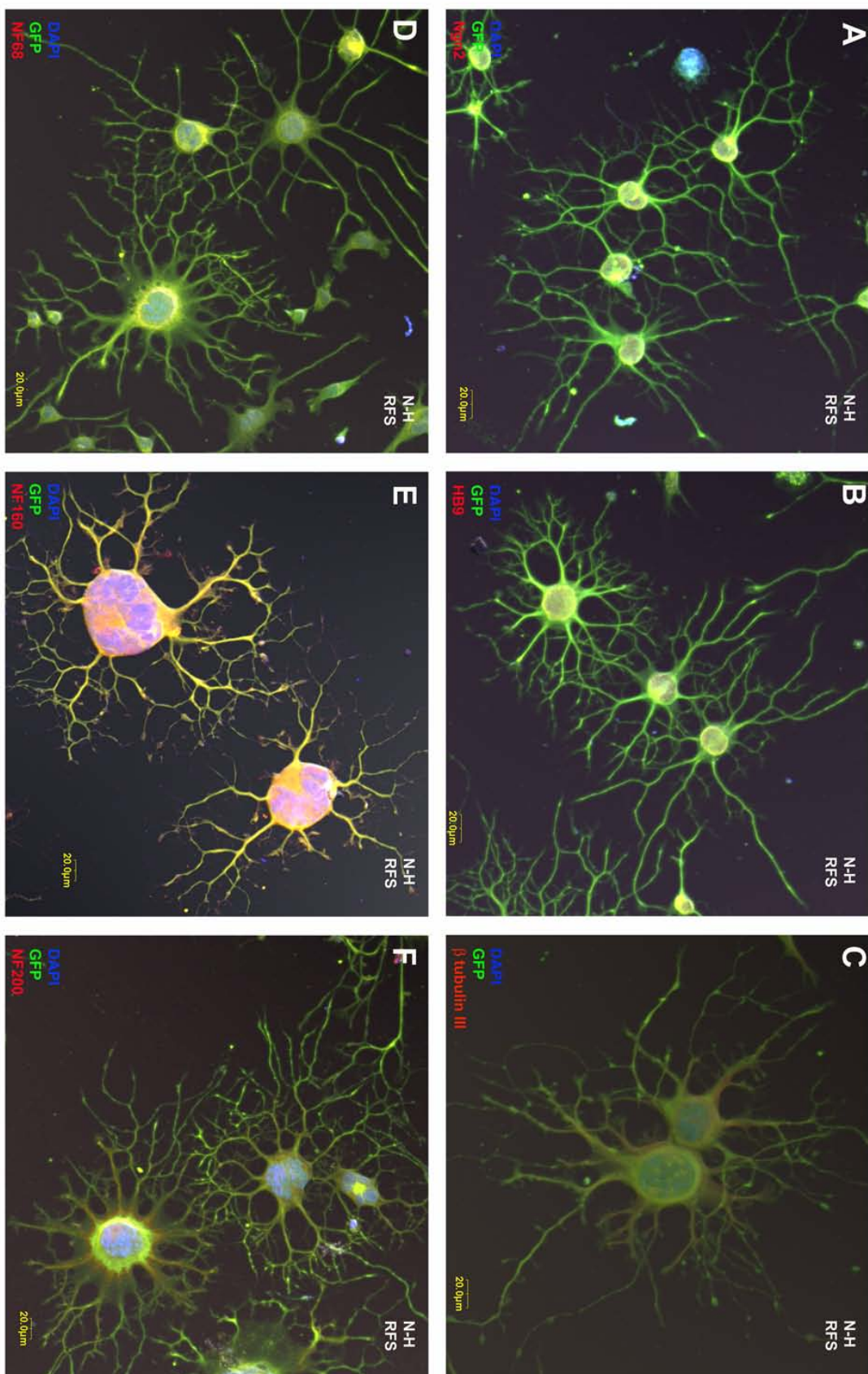


Figure 27. Co-transfection of *Ngn2* and *HB9* increased expression of mature neuronal and motoneuronal antigens after 2 days transfection, 7 days selection and treatment with RFS NSFCs (passage 10-20) exhibited a characteristic more mature neuronal phenotypic expression. These representative micrographs illustrate immunolocalization with GFP (green) (A-F) in *HB9* vectors as well as for several neuronal specific lineage restricted antigens: (A) NeuN (red), (B) ChAT (red), and (C) motoneuron transcription factor Isl1/2 (red). In contrast, (D) nestin (red) expression and (E) BrdU (red) incorporation decreased. Expression of nonneuronal antigens was absent including: (F) MBP (red), (G) GFAP (red), and (H) OX42 (red). DAPI (blue stain for DNA). Confocal images with DIC.

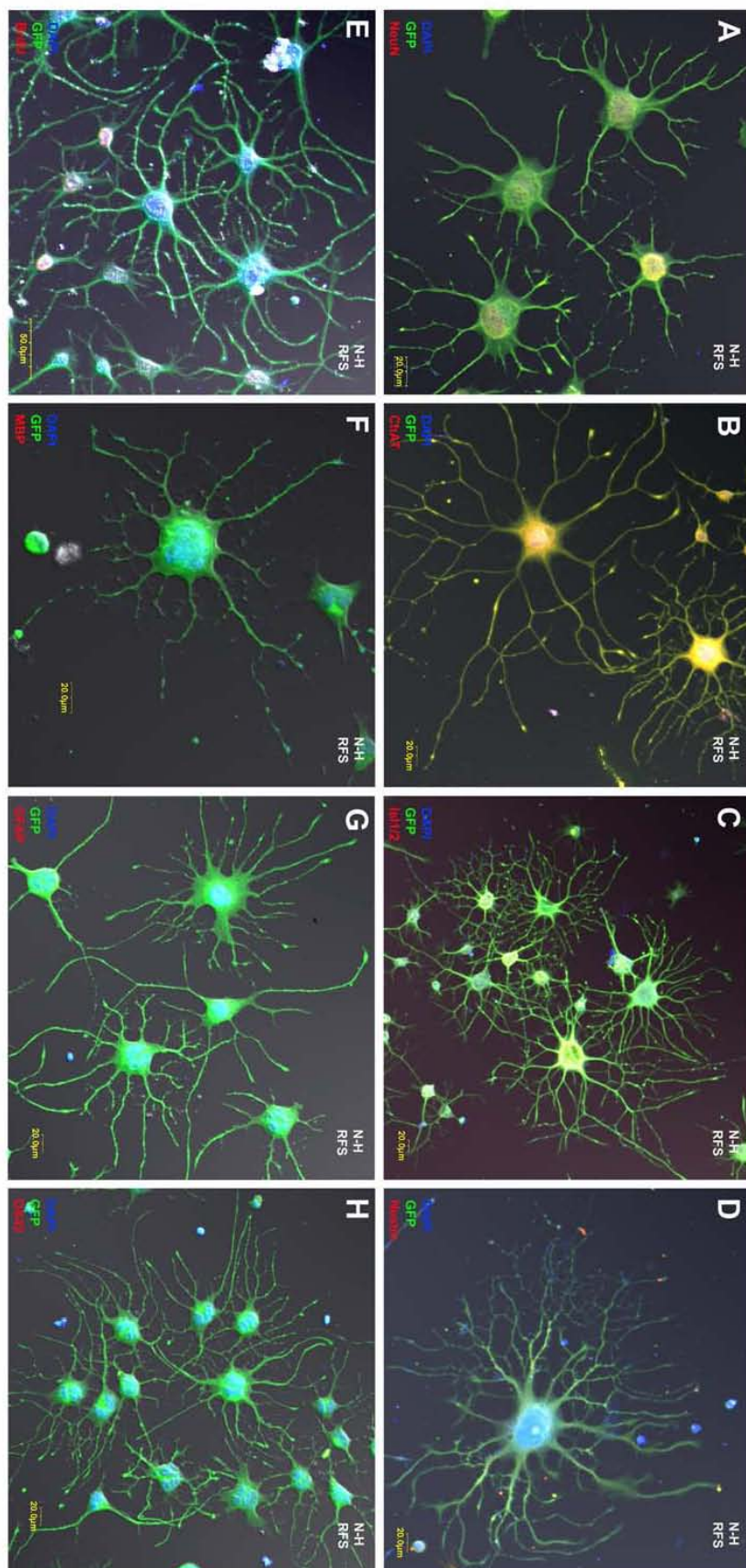


Figure 28. Western blot assay of NSFC (passage 10-20) expression of the transfected cDNA following 2 days transfection and 7 days selection with RFS treatment. Protein samples were separated on SDS-PAGE gels and the expression of Olig2, Ngn2, and HB9 was detected. (A) NSFCs expressed endogenous Olig2 but not Ngn2 or HB9. NSFCs transfected with *Olig2* alone, *Ngn2* alone, *Olig2-Ngn2*, *Ngn2-HB9*, or, *Olig2-HB9*, expressed *Ngn2*. (B) Quantification of protein bands expressed as means \pm standard deviation. NSFCs cultured in defined medium (DFBNM) served as the controls. Actin was used as a control for variation in cell density. The density of the actin band was used as standard to adjust tracing quantification. The pIRES2 expression vector served as the control vector.

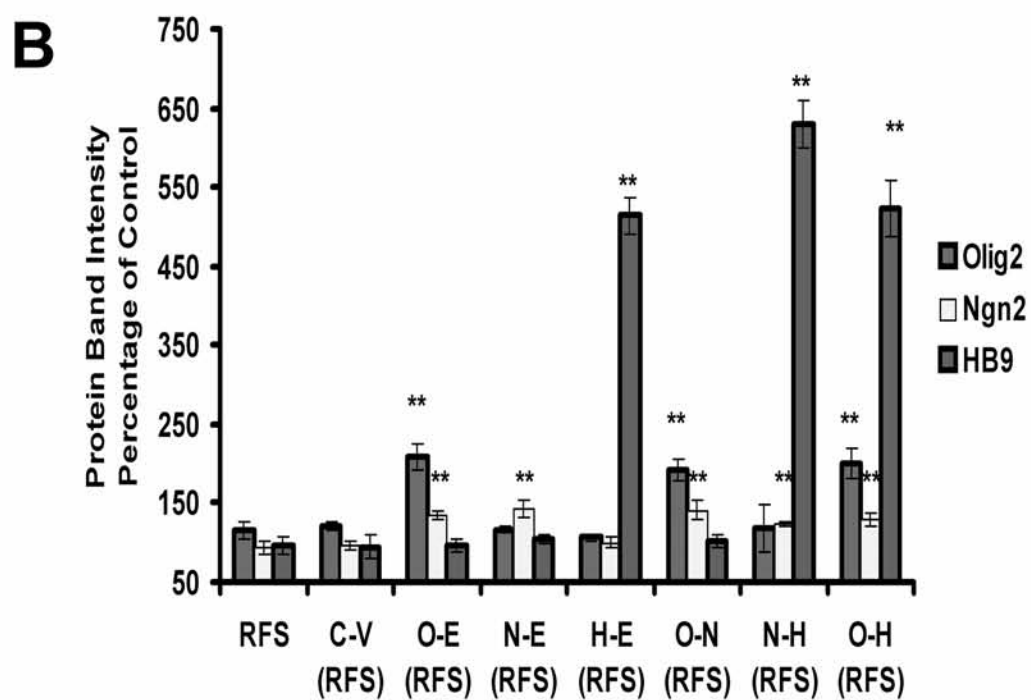
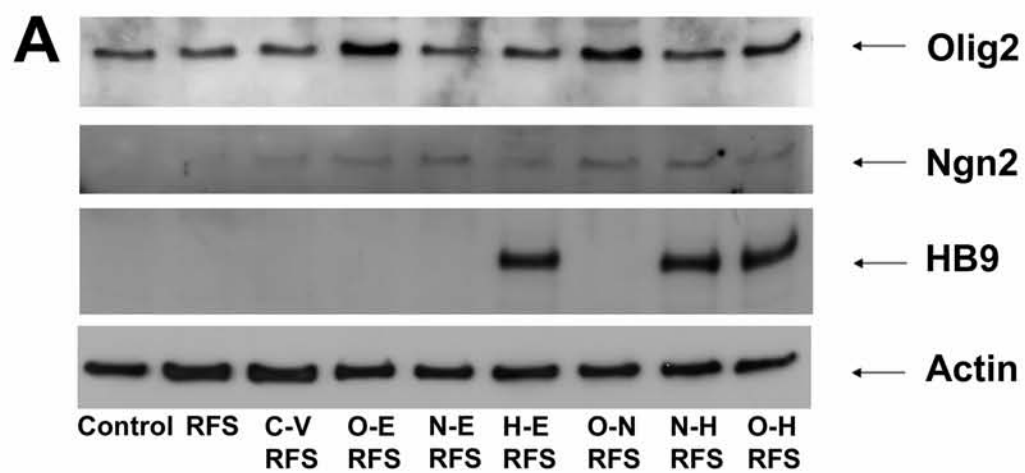


Figure 29. Western blot assay of NSFC expression of neuronal antigens: (A) After 2 days transfection and 7 days selection with RFS treatment, the proteins from NSFCs (passage 10-20) were used to detect the expression of ChAT, VACht, and TH. (B) Quantification of protein bands demonstrated that the expression of ChAT, VACht, and TH were increase among N-H (RFS) and O-H (RFS) groups compared with RFS alone (** $p < 0.01$, t-test). Data were expressed as means \pm standard deviation. NSFCs cultured in defined medium (DFBNM) served as the controls. The pIRES2 expression vector served as the control vector. Actin was used as a control for variation in cell density.

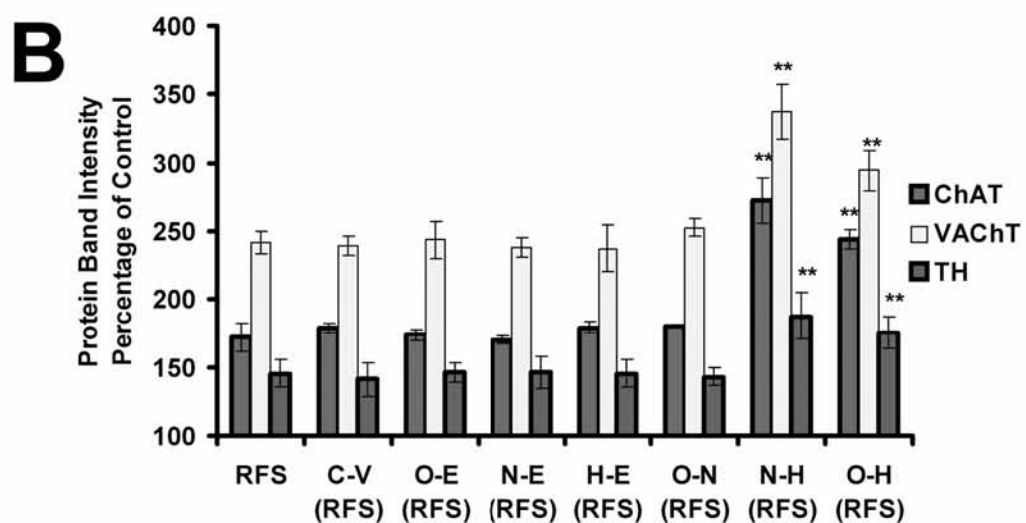
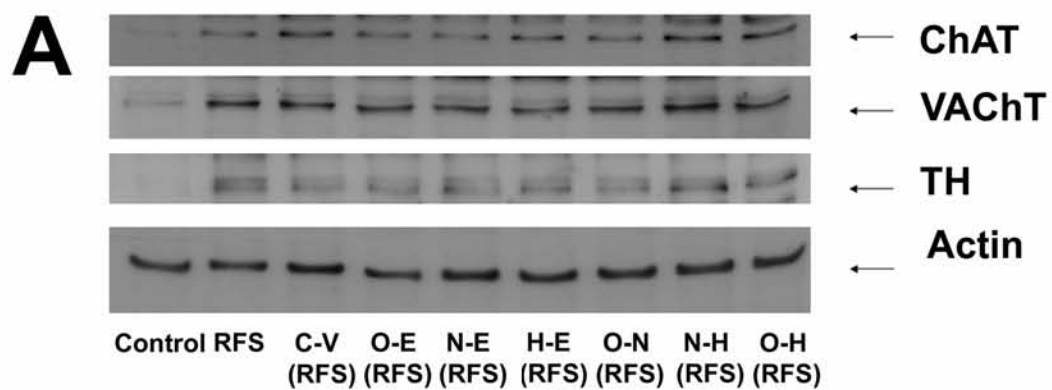


Figure 30. Co-culture studies. The NSFCs (passage 10-20) after 2 days transfection with *Olig2-HB9*; 7 days selection with RFS treatment; expressed (A) *Olig2* (red), (B) *HB9* (red) or (C) motoneuron transcription factor *Isl1/2* (red). (D-H) After 2 days transfection (*Ngn2-HB9* or *Olig2-HB9*) and 4 days selection with RF treatment, NSFCs (passage 11) were seeded onto purified chicken skeletal muscle for 3 days with RS treatment. The NSFCs expressed GFP (green, A-H); (D-H) in co culture neurites were found in frequent contact with muscle straps, where they formed presumptive neuromuscular junctions that expressed (E) ChAT (red), (F) ACh (red), (G) ACh in the muscle strap (red) and (H) synapsin I (red).

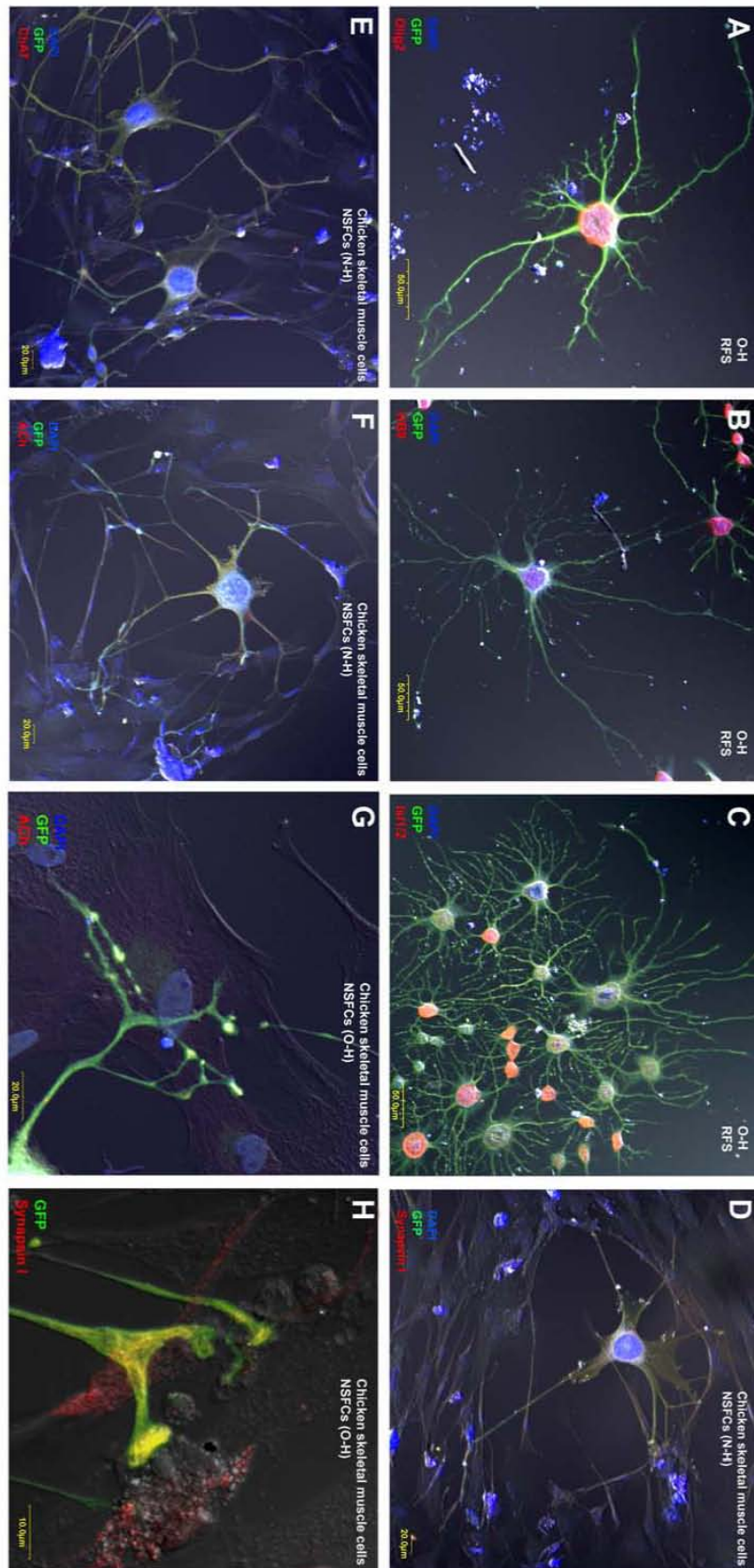


Figure 31. MTT assay. NSFCs (passage 10-20) were cultured in DFBNM without transfection and selection (Control) or transfected with control vector [C-V (RFS)], *Olig2-EGFP* [*O-E* (RFS)], *Ngn2-EGFP* [*N-E* (RFS)], *HB9-EGFP* [*H-E* (RFS)], *Olig2-Ngn2* [*O-N* (RFS)], *Ngn2-HB9* [*N-H* (RFS)], and *Olig2-HB9* [*O-H* (RFS)] for 2 days, selected and treated with RFS for 7 days, and the same number of cells was assayed for viability using the MTT assay. No differences were observed between the groups. Data are expressed as mean \pm standard deviation (SD) (n=12). Each experiment includes triplicate samples. All experiments were repeated a minimum of three times.

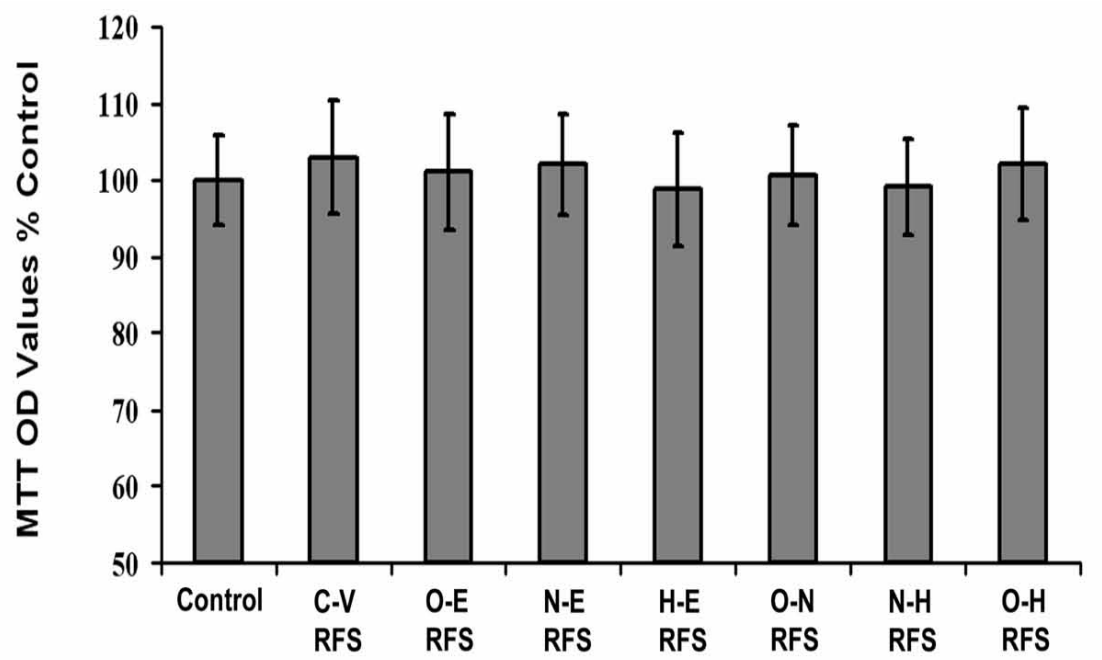
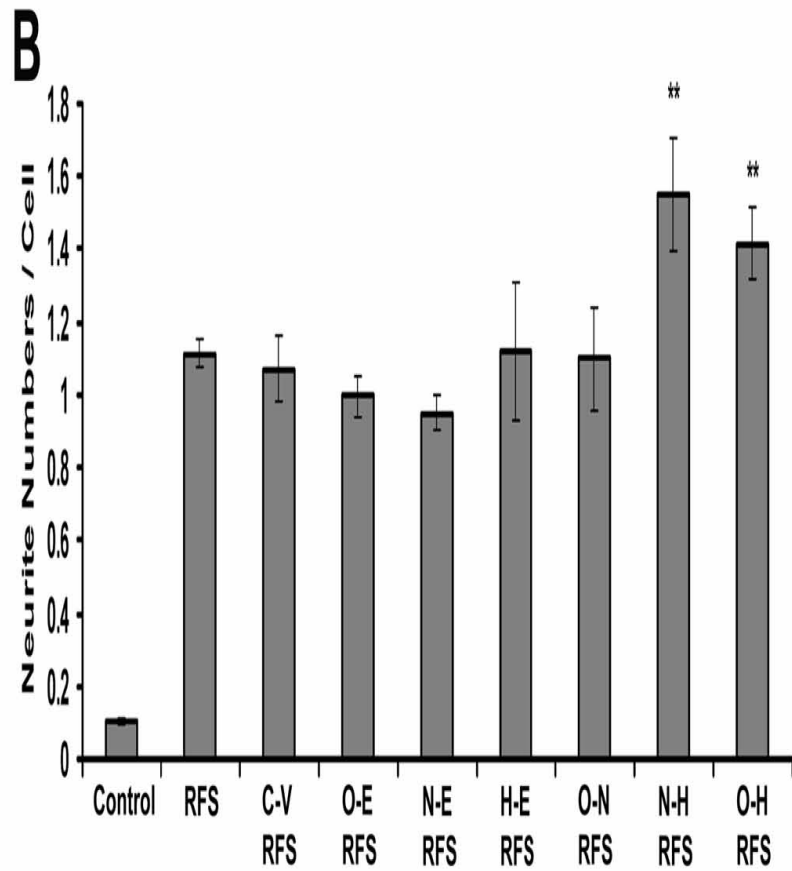
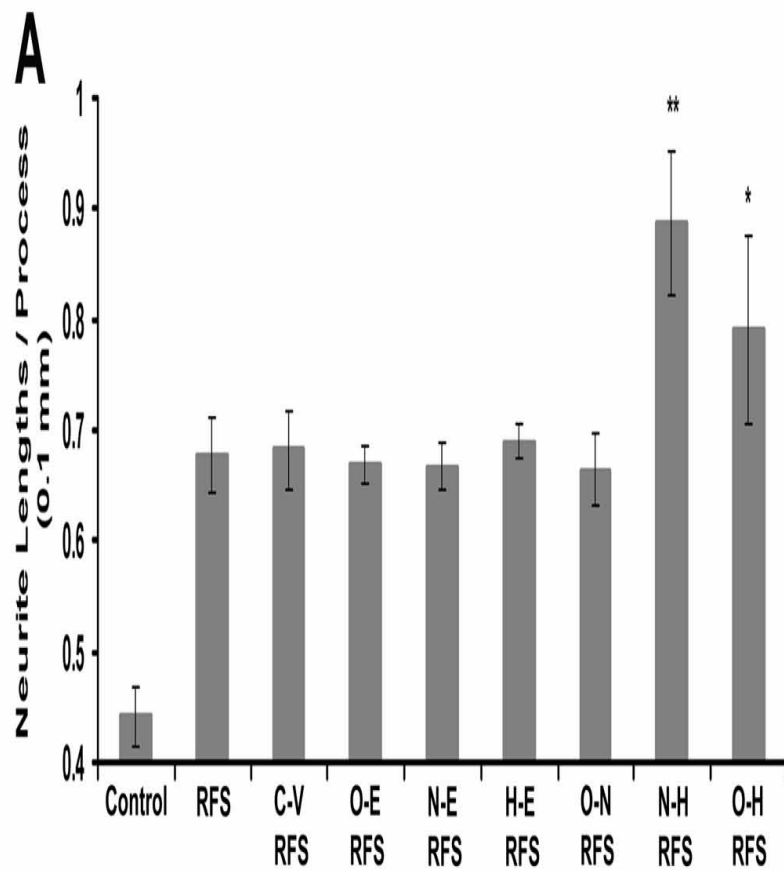


Figure 32. Changes in neurite average lengths (A) and numbers (B) after transfection, selection and RFS treatment. Transfection with *Ngn2-HB9* [*N-H* (RFS)] or *Olig2-HB9* [*O-H* (RFS)] after selection and RFS treatment increased the number and length of neurites per cell compared with RFS(* $p < 0.05$, ** $p < 0.01$, t-test). Values are mean \pm standard deviation.



DISCUSSION

Neural stem cells can differentiate into neurons and glia, and therefore have the potential to provide cell populations for the treatment of various neurological diseases or injuries and for gene function and pharmacological evaluation (Svendsen et al., 1996; McKay, 1997; Gage, 2000). NSFCs isolated from adult human olfactory neuroepithelium remained relatively undifferentiated despite exposure to a variety of media and trophic factors (Roisen et al., 2001; Zhang et al., 2004). This progenitor population appears to have a default characteristic of immature neurons with >97% cells expressing β tubulin III and peripherin, indicating that they may be different from neural stem cells isolated from embryos and/or other species (Zhang et al., 2005; Chandran et al., 2004). However, these NSFCs have the characteristics of neural progenitors (Roisen et al., 2001; Winstead et al., 2005; Marshall et al., in press); they can be driven to differentiate into oligodendrocytes with the transcription factors (Zhang et al., 2005). In this study, NSFCs transfected with *Ngn2-HB9*, or *Olig2-HB9*, and treated with RFS, gradually lost their progenitor characteristics such as nestin expression as previously reported for other cells (Gallo and Armstrong, 1995), and gained the properties of mature neurons.

Recent reports have shown that several key lineage-specific inductive signals play important roles in directing progenitors to differentiate into specific cell types. RA and Shh play key roles in neuronal fate specification in embryonic avian and rodent ventral spinal cord (Diez del Corral et al., 2003; Appel and Eisen, 2003), forebrain (Toresson et al., 1999; Halilagic et al., 2003; Marklund et al., 2004), and hindbrain (Zhang et al., 2003); and the development of dopaminergic neurons in the

midbrain or in the human embryonic stem cells (Perrier et al., 2004). Furthermore, RA interacts with Shh to direct the expression of transcription factors for neuronal differentiation (Novitsch et al., 2003; Diez del Corral et al., 2003; Sockanathan et al., 2003). Consistent with the roles of RA and Shh *in vivo*, there was no change after transfection with the transcription factors in the absence of RFS. Furthermore, RA cooperated with Shh in transfected NSFCs to induce the motoneuron differentiation, consistent with the result that simultaneous exposure of neural cells in embryonic chicken explants to RA and Shh *in vitro* promotes the formation of Olig2+ motoneuron progenitors (Novitsch et al., 2003). Molecular and genetic studies have shown that many transcription factors, when expressed in combination, promote lineage-specific differentiation during embryonic development (Marquardt and Pfaff, 2001). For instance, co-expression of *Olig2* and *Nkx2.2* promoted oligodendrocyte differentiation and maturation *in vivo* and *in vitro*, while expression of either *Olig2* or *Nkx2.2* alone was not sufficient to enable differentiation (Zhou et al., 2001; Zhang et al., 2005). In this study, *Ngn2* and *HB9* have been employed to drive adult human progenitors to differentiate along the motoneuron lineage. Consistent with the previous observations in embryonic neural stem cells, neither *Ngn2* nor *HB9* alone could induce NSFCs to differentiate into neurons. However, NSFCs transfected simultaneously with *Ngn2* and *HB9* exhibited neuronal morphology and antigen expression, resembling *in vivo* patterns. Western blot analysis provided parallel independent assessment to complement the immunological studies. The two different approaches demonstrated that *Ngn2* and *HB9* or *Olig2* and *HB9*, but not *Olig2*, *Ngn2* or *HB9* alone possessed the capability of driving neuronal lineage-specific

differentiation. Consistent with an earlier study that *Olig2* is expressed in avian olfactory epithelium from E11.5 onward (Zhou et al., 2001) a low level of endogenous *Olig2* expression was detected in the NSFCs. This suggests that the low level of endogenous *Olig2* expression was not sufficient to cooperate with *HB9* to induce NSFCs differentiation into neurons or motoneurons. No endogenous *HB9* or *Ngn2* expression was detected.

In the present study, the mechanism of induction of motoneurons by *Olig2* and *HB9* was investigated. Previous studies indicated that *Olig2* determines the motoneuron identity, combined with RA signaling, *Olig2* induces the downstream of *Ngn2* expression as well as other downstream hierarchical transcriptional cascades for the motoneuron differentiation *in ovo* (Novitch et al., 2001; Mizuguchi et al., 2001; Novitch et al., 2003; Diez del Corral et al., 2003). These findings suggested that *Ngn2* may function downstream of *Olig2* and mediate the function of *Olig2* in collaboration with *HB9* to control motoneuronal differentiation and maturation. In agreement with this observation, NSFCs transfected with *Olig2* alone induced *Ngn2* expression. The over expression of *Olig2* and *HB9* in NSFCs achieved equivalent effects as compared to that of *Ngn2* and *HB9*. Expression of *Olig2* in conjunction with *HB9* when combined with the treatment of RFS resulted in increased expression of neuronal and motoneuronal antigens, and neurite formation. NSFCs expressing *Olig2* or *HB9* alone did not exhibit these responses, possibly because the low level of *Olig2* or *Ngn2* was not sufficient to activate downstream transcription factor expression, and a high level of expression may be required to exert its function in concert with other transcription factors such as *HB9* as well as other inductive signals including RA and Shh (Novitch

et al., 2001; Novitch et al., 2003; Diez del Corral et al., 2003). It has also been reported that coexpression of Olig2 and Ngn2 determined motoneuron progenitor cells, resulted in pan-neuronal properties of developing chicken motoneuron differentiation (Novitch et al., 2001; Mizuguchi et al., 2001); and that HB9 is critical for proper motoneuron differentiation in embryonic chicken and mouse spinal cord (Tanabe et al., 1998; Arber et al., 1999; Thaler et al., 1999; Allan and Thor, 2003). In contrast, coexpression of Olig2 and Ngn2, or HB9 alone in NSFCs did not induce the neuronal differentiation, perhaps reflecting differences between species (human and rodent) (Zhang et al., 2005; Chandran et al., 2004), and/or adult and embryonic tissues though key qualitative properties were equivalent (Song et al., 2002).

Previously it has been reported that the murine embryonic stem (ES) cells and mouse P19 embryonic carcinoma cell lines differentiated into neurons and formed synapses after induction by RA. Though there were fewer individual synaptic contacts between pairs of P19 or ES cells, most ES cells had spontaneous synaptic activity (Finley et al., 1996). Synapses have also been reported to form between adult rat hippocampus stem cell-derived neurons and neonatal rat primary hippocampal neurons, although there were some differences in the synapses (Song et al., 2002). It also been reported that synapses formed between rodent neuroblastoma X glioma hybrid cells and mouse embryonic skeletal muscle cells (Christian et al., 1997) and rat pheochromocytoma PC12 cells and L6 rat skeletal muscle cells (Schubert et al., 1977). Others reported that many closely related cell lines failed to form synapses with primary muscle cells *in vitro* (Nelson, 1976; Nirenberg et al., 1983), but did form synapses when transplanted to the cerebellum of newborn mice (Snyder et al.,

1992). In the present study NSFCs transfected with *Ngn2-HB9* or *Olig2-HB9* and treated with RFS expressed more mature motoneuronal markers and appeared competent to form synapses when cocultured with chicken skeletal muscle. Future studies will examine the ability of these transfected cells following transplantation to integrate and form synapses in various regions of rat spinal cord.

In summary, these studies reveal that: (1) the transcription factors (*Ngn2* and *HB9*), which control neuronal, especially motoneuronal development in embryonic chick and rodent CNS, are able to direct adult human olfactory-derived progenitors towards neuronal lineage. (2) In this model, *Ngn2* and *HB9* functioned cooperatively in the environment of a RFS supplement to produce neuronal differentiation. Neither *Ngn2* nor *HB9* alone, or *Ngn2-HB9* in the absence of RFS exhibited phenotypic changes. (3) NSFCs transfected with *Olig2* induced the expression of the transcription factor *Ngn2*. (4) NSFCs transfected with *Ngn2-HB9* or *Olig2-HB9* had an elevated expression of neuronal specific antigens including VACHT, ChAT, NeuN and the motoneuron transcription factor *Isl1/2*. (5) NSFCs transfected with *Ngn2-HB9* or *Olig2-HB9*, and cocultured with chicken skeletal muscle formed multiple contacts that were consistent with the development of neuromuscular junctions.

The readily accessible location of adult human olfactory epithelium makes it possible to obtain endogenous neural progenitors without highly invasive surgery by endoscopic biopsy (Winstead et al., 2005). Our studies suggest that transcription factors can be used to direct these progenitors to differentiate into specific neuronal or glial cell types and thereby expand their therapeutic potential as well as their utility for diagnostic evaluation and genetic studies.

ACKNOWLEDGMENTS

The authors thank George Harding for his assistance with the confocal microscopy. This work was supported by NIH (1920RR15576 to F.J.R.), Kentucky Spinal Cord Head Injury Research Trust (to M.Q.), and National Multiple Sclerosis Society (FA1400-A-1 to J.C.).

CHAPTER VI

SUMMARY

Neural stem cells have been reported to differentiate into neurons, astrocytes, and oligodendrocytes when mitogens are withdrawn or they are exposed to neurotrophic factors (NTFs) including: platelet-derived growth factor (PDGF), ciliary neurotrophic factor (CNTF), T3, glial-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and NT-3 (Svendsen et al., 1996; McKay, 1997; Rao and Mayer-Proschel, 1997; Gage, 2000; Namaka et al., 2001; Dietrich et al., 2002). Adult neural stem cells reside in the CNS as quiescent cells and they need NTFs to proliferate (Fricker et al., 1999; Gage, 2000). The NSFCs derived from adult olfactory neuroepithelium have been characterized as adult human neural progenitors when maintained in serum enriched medium (Roisen et al., 2001). The serum may contain bioactive materials that might mask the effects of exogenous growth factors (Hashimoto et al., 2000). Thus, a defined medium would enable precise culture conditions and permit examination of the effects of exogenous growth factors on the proliferation and differentiation of these adult human neural progenitors. Under the defined experimental conditions, nestin⁺ cells decreased as serum concentration decreased, A2B5⁺ and NCAM⁺ increased in the absence of serum but in the presence of N2 and B27 supplements, there were few mature neural and glial antigens expressed in NSFCs. Furthermore, none of the NTFs evaluated increased NSFCs

viability or lineage restriction. This result is different from that reported concerning other adult neural progenitors, neurotrophic factors EGF, bFGF, NGF, CNTF, BDNF, and GDNF had effects on the proliferation and lineage restriction of embryonic and adult rodent neural stem cells (Cattaneo and McKay, 1990; Reynolds and Weiss, 1992; Svendsen et al., 1996; McKay, 1997; Rao and Mayer-Proschel, 1997; Gage, 2000; Namaka et al., 2001; Dietrich et al., 2002). However, the neurotrophic factors evaluated had no effect on the proliferation and lineage restriction of NSFCs from adult human olfactory epithelium. It may be due to the fact that adult neural stem cells reside in the CNS as quiescent cells and they need NTFs to proliferate (Fricker et al., 1999; Gage, 2000). The olfactory receptors are directly exposed to the environment and may easily be injured. The olfactory epithelial NSFCs differ from other stem cells because throughout life they generate new cells to repopulate the olfactory epithelium every 30-40 days (Caggiano et al., 1994; Graziadei and Graziadei, 1979a; 1979b; Moulton, 1974). Thus, NTFs may not play as pivotal a role in their mitotic activity as other stem cells. The olfactory epithelial NSFCs may be unique since no difference of viability, proliferation, and lineage-restriction was found between the NSFC lines from cadavers, living patients (male, female), or time in culture (Marshall et al., in press). Furthermore, multiple biopsies may be obtained from the same patient at different times if necessary since it was shown that the biopsy procedure did not cause permanent damage to the olfactory epithelium of the patient (Winstead et al., 2005). The difference between human olfactory epithelial NSFCs and neural stem cells from rodent and chicken may be due to species differences and embryonic versus adult sources. It has been reported there were

differences between human and rodent neural precursors in response to fibroblast growth factor 2 (FGF-2) and PDGF during lineage-restriction and differentiation (Chandran et al., 2004), and the difference between adult and embryonic neural stem cells though key qualitative properties were equivalent (Song et al., 2002). The defined media employed in this study, provided the culture conditions for further differentiation into mature oligodendrocytes and neurons.

Recent identification of many key lineage-specific molecules has provided important insights into directing neural progenitors to differentiate into the desired specific cell types. Molecular and genetic studies have shown that many transcription factors, when expressed in pairs, are capable of driving lineage-specific differentiation during embryonic development (Marquardt and Pfaff, 2001). For instance, co-expression of *Olig2* and *Nkx2.2* promoted oligodendrocyte differentiation and maturation *in vivo*, while expression of either *Olig2* or *Nkx2.2* alone was not sufficient to cause such differentiation (Zhou et al., 2001). To further differentiate NSFCs along an oligodendrocyte lineage, several transcription factors were employed. The transcription factors *Olig2*, *Sox10*, and *Nkx2.2* that control oligodendrocytic development in embryonic chick and rodent CNS are able to direct adult human olfactory-derived progenitors towards oligodendrocyte lineage. NSFCs transfected with *Olig2-Nkx2.2* or *Sox10-Nkx2.2* expressed oligodendrocyte-specific antigens, including GalC, CNP, RIP, and human MBP. None of *Olig2*, *Nkx2.2*, *Sox10* alone, and *Olig2-Sox10* showed phenotypic changes. This is the first time that adult human olfactory derived neural progenitors have been shown to respond to factors known to regulate embryonic development resulting in specific lineage restriction.

Furthermore, NSFCs transfected with *Olig2-Nkx2.2* or *Sox10-Nkx2.2* and cocultured with DRGNs formed axonal ensheathments. This is opposite to previous reports that olfactory ensheathing cells (OECs) from mouse failed to myelinate axons of DRG neurons (DRGNs) *in vitro* (Plant et al., 2002). In contrast, others reported that OECs (Devon and Doucette, 1995), oligodendrocytes from mouse embryonic stem cells (Liu et al., 2000), and oligodendrocyte precursor cells from 10-day-old rats (Chan et al., 2004) formed myelin when cocultured with DRGNs. Future studies will address the ability of oligodendrocyte restricted NSFCs after transfection with *Olig2-Nkx2.2* or *Sox10-Nkx2.2* and selection, when transplanted into an animal model of demyelination (ethidium bromide X-irradiation of the ventrolateral funiculus) to enhance remyelination.

The mechanisms underlying neuronal proliferation and differentiation from embryonic neural stem cells or progenitors are under extensive investigation. Neuronal differentiation depends on inductive signals such as neurotrophic factors, retinoic acid (RA), forskolin (FN), and sonic hedgehog (Shh) (Roisen et al., 1972a; 1972b; Singh et al., 1993; Roelink et al., 1994; 1995; Ericson et al., 1997; Chiang et al., 1998; Corcoran and Maden, 1999; Misner et al., 2001; Maden, 2002; Novitch et al., 2003; Diez del Corral et al., 2003; Perrier et al., 2004). The molecular mechanisms through which RA, FN, and Shh mediate cellular differentiation and growth suppression in neural cells are in early stages of study. RA, FN, and Shh signal molecules that control neuronal development in embryonic chick and rodent CNS are able to direct a population of adult human olfactory-derived progenitors towards neuronal lineage including the expression of the mature neuronal marker,

NeuN, motoneuronal transcriptional marker Islet1/2, and tyrosine hydroxylase (an indicator of dopamine production). RA with FN/Shh induced neurite formation of NSFCs; this response was blocked by MEM inhibitor U0126 and PI3-K inhibitor wortmannin but not by PKC inhibitor GF109203X, and PKA inhibitor H89. RA with FN/Shh treated NSFCs, cocultured with chicken skeleton muscle cells gained the capacity to form functional neuromuscular junctions. These are consistent with previous results that RA and Shh play important roles in regulating neuronal specification (such as motoneurons and dopaminergic neurons) in the ventral region of the embryonic CNS and human embryonic stem cells (Sockanathan and Jessell, 1998; Maden, 2002; Novitch et al., 2003; Diez del Corral et al., 2003; Bibel et al., 2004; Roelink et al., 1994; 1995; Ericson et al., 1997; Perrier et al., 2004).

To further differentiate NSFCs into motoneurons, transcription factors *Olig2*, *Ngn2* and *HB9* have been employed to drive adult human progenitors to differentiate along a neuronal lineage. Furthermore RA interacts with Shh to direct the expression of transcription factors for neuronal differentiation (Novitch et al., 2003; Diez del Corral et al., 2003; Sockanathan et al., 2003). Consistent with the previous observations in embryonic neural stem cells, neither *Olig2*, *Ngn2*, *HB9* alone, nor *Olig2*-*Ngn2* could induce NSFCs to differentiate into motoneurons. However, NSFCs transfected simultaneously with *Ngn2*-*HB9* or *Olig2*-*HB9* exhibited neuronal morphology and motoneuronal antigen expression, resembling *in vivo* patterns. In contrast, coexpression of *Olig2* and *Ngn2* or *HB9* alone in NSFCs did not induce neuronal differentiation. These results are different from those reported for embryonic stem cells. Coexpression of *HB9* alone, or *Olig2* and *Ngn2* determined motoneuron

differentiation in embryonic chicken and mouse spinal cords progenitor cells (Tanabe et al., 1998; Arber et al., 1999; Thaler et al., 1999; Novitch et al., 2001; Mizuguchi et al., 2001; Allan et al., 2003). These demonstrate differences between species (Chandran et al., 2004), and between adult and embryonic stem cells (Song et al., 2002).

Future studies will focus on the following issues: First, since transfection efficiency is low due to the heterogeneous population of NSFCs, clonal NSFCs (Othman et al., manuscript in preparation) will be employed to study differentiation via transfection with lineage-restriction transcription factors. Second, since NSFCs after transfection with *Ng2-HB9* or *Olig2-HB9*, selection and RFS treatment, expressed mature neuronal antigen NeuN, and neurotransmitter markers: ChAT, VACHT, and ACh, these NSFCs will be evaluated electrical activity and the ability to conduct and action potential by whole-cell patch-clamp recordings in co-culture with chicken skeletal muscles. Third, when NSFCs were transplanted to the non-injured animals, they survived and migrated (Roisen et al., 2002). The rubrospinal system is BDNF sensitive. So this tract was chosen for use in an animal model of spinal cord injury. When NSFCs were transplanted into an animal model of injured rubrospinal tract, they not only survived and migrated, but also secreted BDNF which promoted rubrospinal axonal regeneration and resulted in functional recovery (Xiao et al., in press). The ability of these transfected cells following transplantation to integrate and form synapses in various regions of rat spinal cord will be examined. Fourth, since these transfected cells increased the expression of TH, these cells will be examined to find optimal conditions to increase the induction efficiency of NSFCs to become TH+,

to determine if they remain viable and TH positive, and finally if they secrete dopamine. The induction of these TH⁺ cells suggests the interesting possibility for their use as an autologous cell source for the treatment of Parkinson's disease in animal models of the disease and in the future as treatment for Parkinson's disease in humans.

Neural progenitors from adult human olfactory neuroepithelium can be obtained without highly invasive surgery via biopsy from the readily accessible location of the olfactory neuroepithelium (Winstead et al., 2005). Our studies suggest that the use of transcription factors to direct adult human olfactory epithelial derived progenitors to differentiate into specific neuronal or glial cell types may provide a range of different populations for autologous transplantation. The therapeutic potential of these populations as well as their utility in studies of gene function and diagnostic application await future studies.

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1993-1994, Suzhou Medical College (now merged in Suzhou University)
Graduate courses study

1985-1989, Nanjing University (Nanjing, Jiangsu, China)
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Teaching Experience

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Research Experience

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